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Award Number: DAMD17-98-1-8510

TITLE: Mechanisms of Mechano-Transduction within Osteoblasts

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REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20030801 029

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

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|---|---|--|---|--|
| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE September 2001 | 3. REPORT TYPE AND DATES COVERED Final (1 Sep 98 - 31 Aug 01) | |
| 4. TITLE AND SUBTITLE Mechanisms of Mechano-Transduction within Osteoblasts | | | 5. FUNDING NUMBERS DAMD17-98-1-8510 | |
| 6. AUTHOR(S) Louis C. Gerstenfeld, Ph.D. | | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Boston University Boston, Massachusetts 02118 E-Mail: lgersten@bu.edu | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | 10. SPONSORING / MONITORING AGENCY REPORT NUMBER | |
| 11. SUPPLEMENTARY NOTES | | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200 Words) <p>Mechanical stimulation is crucial to the homeostasis of adult bone density and mass. The hypothesis of this proposal is that bone cells sense their mechanical environment through specific cell surface receptors (integrins) that interact with specific extracellular matrix (ECM) proteins (osteopontin, bone sialoprotein, and fibronectin) that are the ligands for these receptors. We propose that the expression of these proteins is regulated in response to both cellular interactions with the ECM and mechanical stimulation. Thus, these proteins act like autocrine factors that modify cell behavior in response to changes in either matrix composition or mechanical deformation of the ECM itself. The proposed experiments will define how osteoblasts discriminate the molecular mechanisms by which mechanical signals mediate their actions through the cellular interactions of integrins with the ECM. A determination of the specific integrin isotypes that are involved in the mechano-signal transduction process will be made. The signal transduction system(s) that are responsible for mediating osteopontin, bone sialoprotein and fibronectin gene expression in response to mechanical stimulation, will be determined. Other experiments will examine how aspects of the mechanical stimuli, such as frequency, intensity or duration, effect cell response. Knowledge gained from understanding mechano-signal transduction will facilitate the development of appropriate clinical approaches to enhance the adaptive responses of skeletal tissue to mechanical stimulation.</p> | | | | |
| 14. SUBJECT TERMS | | | 15. NUMBER OF PAGES 89 | |
| | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited | |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

The bone remodeling cycle is known to be intimately involved in the metabolic homeostasis of mineral balance.⁽¹⁾ Bone formation and the remodeling cycle have been shown to be essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected.^(2,3) Bone remodeling has also been hypothesized to provide the means of repairing bone tissue that has been damaged as a result of mechanical fatigue.⁽⁴⁾ Thus, it may be speculated that the skeletal cells (osteoblasts and osteoclasts) which mediate the remodeling process are regulated by their mechanical environment. In order for osteoblasts to respond to their mechanical environment, they must in some way sense it. One mechanism by which cells sense mechanical signals is through the physical deformation of the tissue. The hypothesis of this proposal was that specific cell surface receptors (integrins) that interact with specific extracellular matrix proteins (collagen, osteopontin, bone sialoprotein, and fibronectin) provide the physical link through which mechanical stimuli are transmitted via tissue deformation. As a corollary to this hypothesis, we proposed that the extracellular proteins that are ligands for these receptors are themselves regulated in response to cellular interaction with both the matrix and mechanical stimulation. Thus, these proteins act like autocrine factors that modify cell behavior in response to changes in matrix composition and mechanical deformation, as well as having a structural role in the matrix itself. Maintaining the balance of the extracellular matrix composition within bone then provides a mechanism by which the structural homeostasis of the skeleton may be regulated. This hypothesis and the published data that supports it are found in our review article published in 1999.⁽⁵⁾

The experiments that were carried out with the support of this grant were designed to define how osteoblasts and skeletal cells discriminate mechanical stimuli at a molecular level and how mechanical stimuli and cellular interactions with the extracellular matrix are transduced through integrin receptors and converted to intracellular signals that lead to genomic changes. These experiments defined whether different integrin ligands use common mechanisms in the regulation of their response to these stimuli. A determination of the intracellular second signaling systems (kinases) that are responsible for mediating the altered gene expression of *opn*, *bsp* and *fn* to mechanical stimulation were determined, both by assessing which kinases are activated and through the use of specific inhibitors that block the actions of the different kinase systems. A determination of the specific integrin isoforms that are involved in the different signal transduction processes was made. Other experiments examined if individual gene responses are differentially sensitive to various aspects of the mechanical stimuli (intensity, frequency, duration) or have different thresholds of response to different component parts of the stimuli.

BODY (with Goals as Defined in the Original Proposal)

Goal 1 The first goal of this proposal was directed at defining the molecular mechanisms of signal transduction by which mechanical stimulation regulates the expression of three specific ECM genes (osteopontin, bone sialoprotein and fibronectin) within osteoblasts. These studies tested whether there are common signal transduction pathways that mediate changes in the expression of these genes in response to mechanical stimulation.

- a) The involvement of specific integrin isotypes in the mechano-signal transduction process that mediates these gene's genomic responses to mechanical stimulation was assessed in year 1.
- b) The mechanistic relationship between the signal transduction pathways that are activated by mechanical stimulation vs. cell adhesion was examined in years 2-3
- c) The nature of the second signal transduction pathways that mediate the changes in expression for each of these genes was determined in years 2-3.

Results of Goal 1

The effect of cell adhesion on the expression of these genes was separately examined to determine if this stimuli would mimic the effects of the mechanical stimulation. All three genes showed comparable levels of induction in response to adhesion on the fibronectin coated surfaces in comparison to non-coated surfaces, with maximal levels of induction seen for *bsp* and *opn* at 24 hours after plating while *fn* showed maximal levels of stimulation at 8 hours. Interestingly, while both *opn* and *fn* mRNA expression returned to base line after cell adhesion on fibronectin, *bsp* mRNA levels remained elevated. Examination of the signal transduction pathways that mediated the gene expression in response to attachment on fibronectin coated surfaces showed that both genistein and cycloheximide inhibited the induction of all three genes. This demonstrates that a tyrosine kinase was involved in the cell attachment mediated induction of these genes, and new protein synthesis was a prerequisite to this process. In contrast, the PKA specific inhibitor H-89 only ablated the induction of *fn* expression. Depolymerization of either microtubules or microfilaments with colchicine or cytochalasin D respectively had little effect on the over all expression of these genes in response to cell adhesion, indicating that the adhesion mediated phenomena was not dependent on cytoskeletal integrity. In summary, these results show that both mechanical stimulation and cell adhesion specifically stimulated the expression of integrin binding proteins. These results further demonstrate that while there are common features in the signal transduction processes that mediated the induction of these genes, each gene was separately induced by unique mechanisms. **A complete description of these results is in a manuscript that has been accepted for publication.** Carvalho RS, Schaffer JL, Bumann A and Gerstenfeld LC (2001) "*RGD containing proteins of osteoblasts are responsive to mechanical stimulation and matrix attachment.*" See appended materials.

In our second study, we examined three interrelated relationships pertaining to osteoblast adhesion and the transduction of molecular signals. This study is described completely in appended material and in part below. The first aspect of this study was to define if osteoblasts have variable adhesion properties to different integrin ligands. The second component of the study was to define the relationship between the selective adhesion of the osteoblasts on the different ligands with the induction of specific second signal kinase activities. The final aspect of this study was to determine if osteoblast adhesion to specific integrin ligands would show specific induction of *opn* mRNA expression. The first series of experiments of this study are depicted in Figure 1, panels A and B. These data show that three different extracellular matrix proteins, fibronectin (FN), fibrillar collagen type I (C), and denatured collagen type I gelatin (G), promote selective adhesion, whereas three other proteins, laminin (LN), osteopontin (OPN), and vitronectin (VN), did not. Bovine serum albumin (Al) was used as a control and is included in Panel B. As can be seen from this study, fibronectin was the most effective protein at promoting selective adhesion. Both native and denatured type I collagen also promoted selective

attachment, but unlike fibronectin, both native and denatured collagen showed saturation at between 1 and $3\mu\text{g}/\text{cm}^2$ of surface coating. In contrast, fibronectin did not show saturation in promoting cell adhesion until about $30\mu\text{g}/\text{cm}^2$ (data not shown). These data demonstrate that osteoblast adhesion specifically led to a strong induction of p-tyrosine levels and a generalization of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand. These data then reinforce the conclusions reached in the experiments depicted in Figure 2, which suggest that the induction of intracellular kinase activities are related more to the specific nature of the ligand's interactions with its receptor and less with the process of cellular adhesion alone.

The last set of experiments of this study examined the relationship between osteoblast adhesion on specific ligands and the induction of specific genomic changes, using *opn* mRNA levels as a marker of genomic regulation. These studies are depicted in Figure 4. As can be seen in this figure, the induction of increased *opn* mRNA expression was seen as early as four hours after the cells had been allowed to adhere and continued to increase up to 24 hours. It is interesting to note in this study that once again native fibrillar collagen, fibronectin (*fn*), and to a lesser extent laminin, induced osteopontin (*opn*) gene expression to increase. These results suggest that the same integrin receptors that facilitate specific cell attachment also facilitate the induction of osteopontin expression. It is also interesting to note that neither osteopontin nor vitronectin mediated either of these cell responses, suggesting that they are not facilitated through a $\alpha_v\beta_3$ receptor. The one difference that was observed, however, was in the comparison of cell attachment on denatured collagen versus the induction of osteopontin. This result again suggests that signal transduction through the collagen receptor is only mediated when it interacts with native collagen. **A complete description of these results is in a manuscript that is being prepared for publication.** Carvalho RS, Kostenuick P, Bumann A, Salih E and Gerstenfeld LC (2001) "*Osteoblast adhesion to different integrin ligands selectively mediates osteopontin gene expression.*" (Manuscript in preparation) See appended materials.

Goal 2 The second goal of these studies determined the relative importance of each component part of the mechanical stimuli (intensity, frequency and duration) to the mechano-signal transduction process.

- a) The components of the mechanical stimuli (intensity, frequency, and duration) that is responsible for producing the signal transduction, which leads to the genomic regulation of specific genes in osteoblasts, were examined in year 3.
- b) Short term (<24hr) versus chronic (>72hr) adaptive responses of osteoblasts to variations in duration of mechanical perturbation were defined in year 3.

Results of Goal 2

Towards the completion of goal two, the first series of these experiments was carried out by Emily Samuels, a masters student with the Harvard School of Dental Medicine, and the repeat of these studies was carried out by Anagret Rebin, a masters student from the Dental School of University of Kiel, Germany (on sabbatical). These experiments were focused to determine the component part of mechanical stimuli that would induce changes in osteopontin gene expression. In both sets of studies we analyzed duration and frequency of the mechanical stimulation and examined the effect of fluid flow-induced shear stress on osteopontin expression. Using our

device, which delivers spatially uniform biaxial strain to a membrane surface, osteoblasts were subjected to 1% strain for 1, 5, 15 and 30 minutes and 1, 2, 4 and 8 hours of strain. Analysis of the induction of osteopontin mRNA expression demonstrated that a maximal 145% induction was observed after 8 hours. ($p < 0.05$). Osteoblasts were then subjected to varying frequencies of strain (0.1, 0.25, 0.5 and 1 Hz) for 8 hours. There was clearly a 2-3 fold stimulation in response to increasing frequency again with a significant finding ($p < 0.05$). The effect of fluid induced shear stress was examined in the third part of this study. This was accomplished in the following manner. The membrane was cut into two equal areas that encompassed the inner and outer circular areas of the membrane. The cells were then subjected to an 8 hour period of mechanical stimulation at the same variations in frequency as used in the first part of these studies. Because of the way the device generates its strains, the inner circular area has several fold lower fluid flow than the outer areas. Similarly, higher frequencies will generate higher fluid flows; thus the combination of inner and outer areas with increasing frequency of strain should produce several orders of magnitude greater fluid movement. Analysis of *opn* mRNA production, however, showed that only the highest frequency (1 Hz) generated altered levels of osteopontin expression when comparing the inner and outer areas of the membrane. These results also showed that it was inversely related to the levels of fluid flow. Thus, the inner areas had the higher levels of osteopontin induction. Because a second student repeated these experiments and obtained the same results, we currently believe that the initial results that we obtained are correct. These results, however, are completely opposite to the hypothesis that fluid shear stress was the major mechanical stimulation that facilitated osteopontin (*opn*) expression. These results also would lead us questions as to whether Ca flux through stress activated channels was a component part of the signal transduction mechanisms that induced osteopontin expression as has been suggested by other research groups. This later suggestion, however, has since been shown to be incorrect.⁷ As we moved forward with the third year and finished out these studies, we assessed how to further examine the mechanisms by which strain is effecting osteopontin gene regulation by determining the arrangement of stress fibers and integrin receptor localization within the cells that are found within the various areas of the mechanically stimulated membranes. These studies completed goal two and we anticipate publishing one more manuscript of these our findings.

Continuation of Ongoing and New Studies

New studies that are ongoing and were derived from funding from this application are now directed towards the future long-term goals of extrapolating these model studies into a mammalian system and examining the effects of mechanical stimulation on skeletal stem cell differentiation.

Preliminary mechanical stimulation experiments have been undertaken in C3H10T1/2 murine mesenchymal stem cells and mesenchymal stem cells isolated from murine fracture calluses at 7 days when the tissue is predominantly cartilage. These new experiments have the goal of determining how these cells respond to mechanical stimulation and if mechanical stimulation will change the time course of differentiation that is induced by BMP-7 treatment. Initial experiments will focus on examining osteopontin. Further experiments will assess the markers of both osteogenic and chondrogenic differentiation to determine if mechanical stimulation promotes chondrogenic or osteogenic differentiation selectively. In conjunction with

these in vitro studies, we have established an in vivo model of introducing exact defined micromotion into an osteotomy gap. These experiments are described in "*Induction of a neoarthritis by precisely controlled motion in an experimental mid-femoral defect,*" which was published in the Journal of Orthopaedic Research and is included in the appended materials.

KEY RESEARCH ACCOMPLISHMENTS

1. We have completed all of the experiments outlined in goal one.
2. We have completed the components of goal two and are currently repeating a number of experiments that we initiated in year one.
3. Beyond the listed publications, we will publish one to two more manuscripts from work supported by these studies.
4. We established a new model of in vivo mechanical stimulation to study the role of mechanical forces in promoting chondrogenic differentiation in an osteotomy gap in a rat model.

Reportable Outcomes 2000/2001

Manuscripts, Abstracts, and Presentations

Manuscripts:

- a) Carvalho RS, Schaffer JL, Bumann A and Gerstenfeld LC. (2001) The predominant integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation. J. Cellular Biochem. (in Press)
- b) Cullinane DM, Frederick A, Eisenberg SR, Paccica D, Elaman MV, Lee C, Salisbury K, Gerstenfeld LC and Einhorn T. (2001) Induction of neoarthritis by precisely controlled motion in an experimental mid-femoral defect. J. Orthopaedic Res. (in Press)
- c) Carvalho RS, Kostenuick P, Bumann A, Salih E and Gerstenfeld LC. (2001) Osteoblast adhesion to different integrin ligands selectively mediates osteopontin gene expression. (Manuscript in preparation) (appended in preliminary form)

Abstracts:

Carvalho RS, Kostenuick P, Bumann A, Salih E and Gerstenfeld LC (2001) Selective induction of osteopontin expression in osteoblasts is mediated by different integrin ligands. J. Bone Mineral Res. 16:S1:S:240.

Carvalho RS, Kostenuick P, Bumann A and Gerstenfeld LC. (2002) Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts. Orthopaedic Research Society.

Presentations:

Cullinane D, Frederick A, Gerstenfeld L, Eisenberg S, Pacicca D, Altuwaigi O, Lee C, Salisbury K, Hellenbrand L and Einhorn T (2001) Induction of joint morphology and articular-like cartilage in a bone gap using controlled micromotion. Transactions of Ortho Res. Soc, 26:364

Patents: None

Degrees obtained supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc:

New Animal Model: Dr. Dennis Cullinane, who received partial support from this application, developed a new research area related to mechanical stimulation in bone repair. This new research project is focused on a new model of bone repair in which an external fixator is used to apply controlled micro motion to an osteotomy. Using this surgical approach new joint like structures are able to be formed in a rat model. This approach may have clinical applications for use in the reconstruction of finger joints. This new animal model is now being applied to study cartilage formation.

Funding applied for based on work supported by this award:

- a) Arthritis Foundation; Dennis M. Cullinane Ph.D.
- b) Continuation of funding for this project will be applied for from the NIH in 2002

Employment or research opportunities applied for and/or received on experiences/training supported by this award:

a) In the last year two M.D./Ph.D. candidates have been recruited into this laboratory to work on areas of biomechanical stimulation of bone healing and bone cell response. Ms. Kristy Salisbury, a bioengineering student, will be working on the new project area of joint regeneration by biomechanical stimulation. Mr. Alexios Apazidis, a biochemistry student, will be continuing work using cultured cells and mechanical stimulation. In the coming year we will be assessing effects of mechanical stimulation on mesenchymal stem cells and cultured cells from fracture callus tissues as we rewrite this project for NIH funding.

CONCLUSIONS

The major conclusions from this proposal are as follows:

1. Integrin ligands as a class are induced in osteoblasts in response to mechanical stimulation and adhesion. Such findings provide strong evidence to support the hypothesis that these molecules act like autocrine or paracrine factors.
2. Cell adhesion of osteoblasts is specifically mediated by $\beta 1$ class of integrins. This same class of integrins appears to be responsible for the signal transduction process that stimulates osteopontin induction. The $\beta 3$ integrin ligands vitronectin and osteopontin neither mediate

specific adhesion nor induce osteopontin gene expression, but they do selectively induce specific kinase activities.

3. Component aspects of the mechanical stimulation do affect the induction of the gene. Both greater duration and increasing frequency clearly appear to enhance response to the mechanical signal. Shear stress induced by fluid flow does not appear to be a mediating factor in osteopontin induction in response to mechanical stimulation.

FINAL REPORT BIBLIOGRAPHY

1. Gerstenfeld, LC. (1999) Osteopontin in skeletal tissue homeostasis: an emerging picture of the autocrine/paracrine functions of the extracellular matrix. *Research Prospective, J. Bone and Mineral Research*, 14:850-855
2. Carvalho RS, Schaffer JL, Bumann A and Gerstenfeld LC. (2001) The predominant integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation. *J. Cellular Biochem.* (in Press)
3. Cullinane DM, Frederick A, Eisenberg SR, Paccica D, Elaman MV, Lee C, Salisbury K, Gerstenfeld LC and Einhorn T. (2001) Induction of neoarthrosis by precisely controlled motion in an experimental mid-femoral defect. *J. Orthopaedic Res.* (In Press)
4. Carvalho RS, Kostenuick P, Bumann A, Salih E and Gerstenfeld LC (2001) Osteoblast adhesion to different integrin ligands selectively mediates osteopontin gene expression. (Manuscript in preparation)
5. Samuel, E J. Osteoblast induction of osteopontin expression: response to changes in duration and frequency of mechanical strain, and to variation in flow induced shear stress. Masters thesis, Harvard School of Dental Medicine, Copywrite Harvard School of Dental Medicine.

Abstracts:

Carvalho RS, Kostenuick P, Bumann A, Salih E and Gerstenfeld LC. (2001) Selective induction of osteopontin expression in osteoblasts is mediated by different integrin ligands. *J. Bone Mineral Res.* 16:S1:S:240

Carvalho RS, Kostenuick P, Bumann A and Gerstenfeld LC. (2002) Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts. Orthopaedic Research Society.

Presentations :

Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts. Presented at "Osteoporosis as a

failure of bone's adaptation to functional load bearing." The Wellcome Trust Foundation, Highgate, England 1999.

Final List of Personnel:

Louis C. Gerstenfeld, Ph.D.
Paul Kostenuick, Ph.D.
George L. Barnes, Ph.D.
Dennis Cullinane, Ph.D.
Irene Simkina, M.S.
Bohus Svagar, M.D.
Colleen Keyes, B.S., M.Sc.
Johanna Cruceta, B.S.
Thomas Dellatorre, B.S.
Michael Elman, B.S.
Erjan Salih, Ph.D. (Subcontract)

REFERENCES:

1. Jilka RL, Manolagas SC 1994 In *Osteoporosis* Chapter 2 The cellular and biochemical basis of bone remodeling. Pg 17-47 ed. R. Marcus, Blackwell Scientific Publications, Cambridge MA
2. Lanyon LE, Goodship AE, Pye C, McFie H 1982 Mechanically adaptive bone remodeling. *Biomechanics* **17**: 897-906.
3. Lanyon LE, Rubin CT 1987 Functional strain on bone tissue as an objective and controlling stimulus for adaptive bone remodeling. *J Biomech* **15**:141-154.
4. Mori S, Burr DB 1993 Increased intracortical remodeling following fatigue damage. *Bone* **14**:103-109.
5. Gerstenfeld LC 1999 Editorial\Perspective Osteopontin in skeletal tissue homeostasis: an emerging picture of the autocrine\paracrine functions of the extracellular matrix. *J Bone Miner Res* **14**:850-855.
6. Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, Duncan RL 1997 Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. *Am J Physiol* **273**:C810
7. Chen NX, Ryder KD, Pavalko FM, Turner CH, Burr DB, Qiu J, Duncan RL. 2000 Ca²⁺ regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts. *Am J Physiol Cell Physiol* **278**:C989-97



Radiation Protection
Office

88 East Newton Street, D-604
Boston, Massachusetts
02118-2394
617 638-7052

To: Dr. Gerstenfeld

Date: June 24, 1998

From: Victor Evdokimoff, Secretary Radioisotope Committee *VNE/CW*

Subject: Authorization to use radioisotopes at BUMC

On June 24, 1998 your application X, renewal ____, amendment ____ to use radioisotopes at BUMC was approved. You are only authorized for the following isotope(s), quantities, etc.

| <u>Isotope(s)</u> | <u>Form</u> | <u>Max./order</u> | <u>Max./year</u> | <u>Possession limit</u> |
|-------------------|--|-------------------|------------------|-------------------------|
| H-3 | Amino acid, Nucleotide | 5 mCi | N/A | N/A |
| P-32 | Phosphorous, Nucleotide | 5 mCi | N/A | N/A |
| C-14 | Chloramphenicol, Amino acids | 1 mCi | N/A | N/A |
| S-35 | Na Sulfate, Nucleotide, Amino acids | 1 mCi | N/A | N/A |
| P-33 | Phosphorous, Nucleotides | 1 mCi | N/A | N/A |

Your authorization code number is G-16. This number must appear on all requisitions for isotopes. In addition, the following conditions apply to your authorization:

- 1) The Radioisotope Committee is recommending you consider alternatives to using $^3\text{H}/^{14}\text{C}$ such as non-radioactive tracers.
- 2) All personnel under your permit planning on using P-32 must complete the individual training requirements. In addition, any user of 1 mCi or more of P-32 at one time is required to be monitored by TLD. Please contact the RPO for assistance.



Boston University
Medical Campus

Office of Research
Administration

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BOSTON UNIVERSITY MEDICAL CENTER

Institutional Biosafety Committee

Renewal Letter: Biohazard Project

IBC Coordinator: *Mary Gistis*
Mary Gistis

Principal Investigator: Dr. Louis Gerstenfeld

Project Title: Musculoskeletal Research

Approval Number: # A-168

Renewal Date: November 4, 1998

Containment Level: BL-2/Universal Precautions

Comments: related to rDNA projects 497, 500, 501

For your records, make several copies of this document to avoid delays when filing your grant applications with federal agencies.

IBC APPROVAL LETTER



Boston University
School of Medicine

Institutional Animal
Care and Use
Committee

700 Albany Street, W707
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Louis C. Gerstenfeld PhD
Associate Professor
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Boston MA 02118

7/11/2000

RE: Application No. 98-096

Agency: Department of the Army

Title: Mechanisms of Mechano-Transduction Within Osteoblasts

Protocol Status: APPROVED, 7/22/1998

ANIMAL NUMBERS/YR. 1248 chicken embryos/year x 4 yrs

BIOHAZARDS:

Dear Dr. Gerstenfeld

Your application for use of animals in research or education has been reviewed by the Institutional Animal Care and Use Committee at Boston University Medical Center. The protocol is APPROVED as being consistent with humane treatment of laboratory animals and with standards set forth in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

The Laboratory Animal Science Center at Boston University Medical Center has been accredited by the American Association for Accreditation of Laboratory Animal Care since 1971. Boston University Medical Center has had an Animal Welfare Assurance on file with the Office for Protection from Research Risks (OPRR) since January 1, 1986. Boston University's Animal Welfare Assurance number is A-3316-01.

Animal protocols may be approved for up to three years. However, if the study extends beyond one year from the approval date, an annual continuation form (1 page) must be submitted. If a project is to extend beyond three years, a full application must be resubmitted and reviewed at the end of the initial three year period.

Sincerely,

A handwritten signature in cursive script, appearing to read "Colleen A. Cody".

Colleen A. Cody, Coordinator
Institutional Animal Care and Use Committee

c: Wayne W. LaMorte, M.D., Ph.D., M.P.H. Chairman, IACUC
Veterinary Staff, LASC

Appendix A

“Selective Induction of OPN Expression in Osteoblasts is Mediated by Different
Integrin Ligands”

R.S. Carvalho, P.J. Kostenuik, A. Bumann, E. Salih, L.C. Gerstenfeld

Journal of Bone and Mineral Research, Vol 16, Suppl 1, Sept 2001, pp S240
Abstract SA052

Final Report

DAMD17-98-1-8510

Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

P.I., Louis C. Gerstenfeld, Ph.D.

Selective Induction of OPN Expression in Osteoblasts is Mediated by Different Integrin Ligands. R. S. Carvalho^{*1}, P.J. Kostenuik^{*2}, A. Bumann^{*3}, E. Salih^{*4}, L.C. Gerstenfeld¹
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Bone remodeling is partly regulated by the mechanical environment of skeletal cells. However, some forms of signals derived from mechanical stimulation are only perceived by cells after adhesion has taken place. Therefore, signal transduction and gene expression events activated by the latter may be similar, if not identical, to those induced by integrin mediated cell adhesion. Recent studies have shown that osteopontin (OPN) mediates osteoblastic and osteoclastic interaction with the mineralized matrix. Thus, OPN is both associated with remodeling and formation. These studies examined whether osteoblasts discriminate cellular interactions with ECM ligands to mediate *opn* mRNA expression. Embryonic chicken calvaria osteoblasts were plated using identical cell numbers in plates coated with fibronectin (FN) (1mg/ml), collagen type I (Col1) (1mg/ml), denatured collagen type 1 (gelatin-G) (1mg/ml), osteopontin (OPN) (1mg/ml), vitronectin (VN) (1mg/ml), laminin (LN) (1mg/ml) or poly-L-lysine (pLp). Subsequently, a determination of the intracellular second signaling systems (protein kinases A and C) that are responsible for mediating the *opn* gene expression to cellular adhesion were determined. Finally, we evaluated the intracellular distribution of focal adhesion kinase (FAK), p-tyrosine (PT) and vinculin (VC) on surfaces coated with FN or pLp. Results indicated that FN generated the strongest induction of OPN expression, followed by Col1 and LN. While G was weakly inducing, neither OPN, pLp or VN was capable of inducing *opn* mRNA expression, suggesting that the latter molecules did not facilitate specific cell adhesion. Induction of PKA and PKC occurred concomitantly only in osteoblasts from FN-coated dishes, while the other ligands promoted PKA solely. Thus, specific effects on kinase activities appear to be dependent on the selective ligand interactions, which are distinct from that of cell adhesion alone. Similarly, osteoblast adhesion did not appear to alter the general intracellular localization of FAK or VC. By contrast, a strong induction was noted for PT with generalized changes of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand. Taken together with the *opn*-mediated gene expression results, these data suggest that induction of intracellular second signal kinase activity are related to the specific nature of the ligand's interaction with the receptor and less with the process of cellular-mediated adhesion alone.

Appendix B

“The Predominant Integrin Ligands Expressed by Osteoblasts Show Preferential Regulation in Response to both Cell Adhesion and Mechanical Perturbation”

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Accepted for publication in the Journal of Cellular Biochemistry, 2001

Final Report

DAMD17-98-1-8510

Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

P.I., Louis C. Gerstenfeld, Ph.D.

Journal of Cellular Biochemistry

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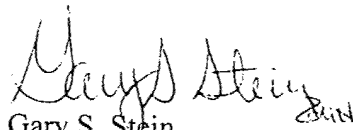
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THE PREDOMINANT INTEGRIN LIGANDS EXPRESSED BY OSTEOBLASTS SHOW PREFERENTIAL REGULATION IN RESPONSE TO BOTH CELL ADHESION AND MECHANICAL PERTURBATION

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Running title: RGD-proteins, integrins and mechanical perturbation

Supported by a grant from the Department of Defense Bone Health and Military Readiness Program
DAMD17-98-1-8510

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Summary: Previous studies demonstrated that both mechanical perturbation and cell adhesion induced the expression of osteopontin (*opn*) by osteoblasts [Carvalho et al., 1998]. The present study examined if these same stimuli on osteoblasts would induce the expression of other integrin binding proteins, specifically fibronectin (*fn*) and bone sialoprotein (*bsp*). All three genes showed 3 to 4 fold maximal induction in response to both cell adhesion and a single two hour period of an applied spatially uniform, dynamic biaxial strain of 1.3% at 0.25 Hz . Each gene, however, responded with a different time course of induction to mechanical strain, with *bsp*, *fn* and *opn* showing their maximal response at one, three and nine hours respectively after the perturbation period. In contrast, peak induction to cell adhesion was observed at 24 hours for *bsp* and *opn*, while *fn* levels peaked at 8 hours. Interestingly, while both *opn* and *fn* mRNA expression returned to base line after cell adhesion, *bsp* mRNA levels remained elevated. Examination of collagen type I and osteocalcin mRNAs showed unaltered levels of expression in response to either type of perturbation. A common feature of the signal transduction pathways, which mediate the gene expression in response to both cell adhesion and mechanical perturbation, was the activation of specific tyrosine kinases based on the ablation of the induction of these genes by the tyrosine kinase inhibitor genistein. While cycloheximide blocked the induction of all three mRNAs in response cell adhesion, it failed to block induction of any of these genes in response to mechanical perturbation. Such results suggest that the induction of these genes after mechanical perturbation was mediated by an immediate response to signal transduction, while cell adhesion mediated effects secondary to signal transduction. Depolymerization of microfilaments with cytochalasin D had no effect on the overall expression of any of these genes in response to cell adhesion and only blocked the induction of osteopontin expression in response to mechanical perturbation. These results suggest that cytoskeletal integrity is only selectively important in the signal transduction of certain types of stimuli and for the regulation of certain genes. In summary, both mechanical perturbation and cell adhesion

specifically stimulated the expression of integrin binding proteins. Furthermore, while there are common features in the signal transduction processes that mediate the induction of these genes in response to both stimuli, specific genes are separately regulated by specific mechanisms that are unique to both forms of stimuli.

INTRODUCTION

The mediation of cellular responses to mechanical stimuli depends in part on the recognition and interaction of selected cell-surface receptors with the extracellular matrix. Integrins are one class of cell surface receptors that mediate cell adhesion to the extracellular matrix. The integrins are a family of heterodimeric membrane receptors composed of multiple α and β isoforms. Variations in the extracellular domains of the individual isoforms of integrin receptors impart their specificity for specific extracellular matrix proteins [Hynes, 1992; Miyachi, 1995]. The interaction of many integrin isoforms with their specific ligands is mediated by their recognition of the amino acid sequence arginine-glycine-aspartic acid-serine (RGDS) [Hynes, 1992]. It has been demonstrated that the binding of specific adhesion proteins to their integrin receptors generates a cascade of intracellular signals that are responsible for the regulation of a wide variety of cellular responses [Damsky and Werb, 1992]. Such interactions facilitate the appropriate functioning of essential cell functions such as cell adhesion, cell migration and survival of many cell types. These many functions are in turn mediated through integrin receptor activation of specific signal transduction mechanisms or by their mediation of structural alterations in the cytoskeletal architecture of cells [Juliano and Haskill, 1993].

In bone tissue, osteoblasts express high levels of several different RGD-containing proteins, the most predominant being osteopontin, bone sialoprotein and fibronectin [Gotoh et al., 1995; Puleo and Bizios, 1992; Winnard et al., 1995]. Osteopontin has been shown to interact with both osteoblasts and osteoclasts [Oldberg et al., 1988; Gotoh et al., 1990; Ross et al., 1993], and it is thought to play a role in mediating osteoclast resorption of bone tissue [Reinholt et al., 1990; Denhart and Guo, 1993]. The

expression of osteopontin is seen concurrently with alkaline phosphatase, and it has been identified as an early marker of osteoblast differentiation [Gerstenfeld et al., 1990]. Bone sialoprotein is another specific integrin ligand that is expressed by osteoblasts having a very restricted expression to only cells within the skeletal lineage and is seen predominantly in areas of mineralized growth cartilage and osteoid [Chen et al., 1994; Yang and Gerstenfeld, 1996; Yang and Gerstenfeld, 1997]. Bone sialoprotein has been shown to initiate calcification through its binding properties to collagen, calcium and hydroxyapatite [Hunter and Goldberg, 1994]. Unlike the former two proteins, fibronectin is expressed ubiquitously in most connective tissues. However, this protein appears to play an important role in the mechanisms of cell attachment, spreading and migration during early osteoblast differentiation [Winnard et al., 1995]. In addition to the RGDS peptide, fibronectin contains a synergistic adhesion site to the RGD sequence. It also contains two adhesion sites in the type III connecting segment of the whole molecule, the CS1 portion and the arginine-glutamic acid-aspartic acid-valine (REDV) sequence within the CS5 portion of fibronectin [Puleo and Bizios, 1992]. Some studies suggest that fibronectin also acts as an activator of cell adhesion rather than as a direct adhesion molecule [Curtis et al., 1992]. Thus fibronectin may mediate multiple interactions and responses by cells. Currently, there is a large body of data to suggest that the RGD-containing proteins osteopontin, bone sialoprotein and fibronectin play essential roles in the cellular differentiation and migration of osteoblasts during skeletal growth and/or in the initiation of spatial deposition of mineral in the extracellular matrix [Curtis et al., 1992; Hunter and Goldberg, 1993; Gerstenfeld et al., 1995; Schaffer et al., 1996].

Previous studies have shown that both the mechanical environment of osteoblasts and cell adhesion induce osteopontin gene expression [Toma et al., 1997; Carvalho et al., 1998]. These studies suggest that the interactions of cells with the extracellular matrix are integral components to the mediation of these stimuli. However, cellular perturbation through receptors may occur both through

the occupancy of the receptor as well as deformation through the engagement of the receptors with matrix attachment [Miyachi et al., 1995]. Furthermore, it has been shown that specific reverse phosphorylation of specific proteins appears to regulate various intracellular pathways exclusively upon cellular attachment [Guan et al., 1991]. These results raise the possibility that signal transduction may be dependent on the matrix composition and that the cellular matrix components themselves may function as autocrine factors that regulate their own expression [Gerstenfeld, 1999]. Mechanical perturbation through receptor deformation and stimuli through receptor-ligation then may share overlapping molecular elements that mediate intracellular signal transduction and lead to common genomic responses. Such modulations may also involve cytoskeletal integrity and its relation with the integrin receptors. It has been hypothesized that cellular shape changes determine signal transduction pathways through the direct deformation of cellular membranes and reorientation of the microfilament network, thereby affecting integrin behavior [Ingber, 1991].

In previous studies from our laboratory, we have shown that that the continuous application of a dynamic, spatially uniform biaxial mechanical perturbation to osteoblasts leads to cytoskeletal rearrangement as well as changes in the extracellular matrix composition cellular response [Meazzini et al., 1998]. Other studies have shown that mechanical strain in cells of osteoblastic lineage only showed increased DNA synthesis when the cells were attached to specific ligands such as fibronectin [Wilson et al., 1995]. Thus, it seems that mechanical perturbation leads to both the physical alteration of ECM proteins and alterations in cellular architecture, which suggests that cells reach a homesostatic balance that is regulated through a complex set of receptor mediated interactions between the cells and the extracellular matrix proteins. In this study we examined if genes such as *opn*, *bsp* and *fn*, which specifically interact with integrins, would be commonly induced following either mechanical perturbation and/or cell-ligand binding. It was further examined if there are common signal transduction

pathways by which the expression of these proteins were modified following cellular attachment or mechanical perturbation, and if these pathways are unique or have overlapping mechanisms.

MATERIALS AND METHODS

Materials

All tissue culture supplies, cytochalasin D, colchicine, and cycloheximide were from Sigma Chemical Company (St. Louis, MO). H89 genistein was from LC Laboratories (Woburn, MA). Nylon membranes for Northern blots were from Biotrans, ICN Corp. (Aurora, OH).

Cell Culture

Seventeen-day embryonic chicken calvaria osteoblasts were and grown in culture as previously described [Gerstenfeld et al., 1988]. These cells were plated at a density of 2×10^6 cells in 100 mm tissue culture dishes either left uncoated or coated with purified fibronectin (1mg/ml) as previously described [Schaffer et al., 1994]. Cultures were grown for two weeks until they reached confluence in minimum essential media supplemented with 10% fetal bovine serum (FBS). The medium was changed to BGJ_b supplemented with 10% FBS with the addition of 10mM β -glycerophosphate and 12.5 μ g/ml ascorbic acid. All analyses were performed on at least three separate preparations of cells, and all data are presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the standard deviation (SD) of the determinations from separate experiments and the number of replicates that were used for each measurement is denoted in each figure.

Mechanical Perturbation / Attachment Assays

The mechanical stretch apparatus used for these experiments was as previously described [Schaffer et al., 1994]. The design of the device imposes a verified temporal and spatial displacement profile to an optically transparent elastomeric membrane in which the strain magnitude was experimentally demonstrated to be homogeneous and isotropic (i.e. radial strain = circumferential strain

= constant over the culture surface) [Schaffer et al., 1994]. A polyurethane membrane (a generous gift of Dow Chemical Corporation, Midland, MI) was used in the culture dishes allowing for a constant 1.3% uniform biaxial strain at 0.25 Hz to be applied for a single two hour period. For each experiment, non-stimulated controls were performed on identical culture surfaces at the same time and from the same preparation of cells grown at identical conditions as the mechanically stimulated cultures. In all experiments for mechanical stretch, determinations were carried out six hours after the end of the two hour period of active cellular perturbation. For the attachment/integrin ligation assays, the cells were allowed to attach to fibronectin coated (1mg/ml) dishes, at the same concentration as those of mechanical stimulated cultures, for 24 hours. Fibronectin served as the basic ligand and uncoated plastic plates served as controls.

Signal Transduction Studies

Signal transduction pathways that mediate the cell responses of the mechanical perturbation and/or attachment/ligation were investigated by the use of specific chemical inhibitors. The final concentration for each of these compounds was: 50 mM cycloheximide, 20 mg/ml genistein, 1 μ M H89 (Sigma), 50 μ M cytochalasin D (Sigma) and 1 μ M colchicine (Sigma). Cycloheximide and genistein were incubated for 30 minutes, while cytochalasin D was incubated for 1 hour and colchicine for 6 hours. Controls were separately determined for each compound in cultures treated identically with the various compounds but in which the cells were either not mechanically stimulated or were attached to uncoated dishes.

Isolation and Analysis of RNA

Total RNA was isolated using tri-ReagentTM (Molecular Center, Cincinnati, OH) according to the manufacturer's instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde [Toma et al., 1997] and 5 mg of total RNA was loaded per gel/lane. Chicken cDNA's used for these studies were pro α 1[I] collagen [Lehrach et al., 1979], osteocalcin [Nuegelbauer et al., 1995],

osteopontin [Moore et al., 1991] and bone sialoprotein [Yang et al., 1995]. Northern blots with ³²P cDNA-labelled probes were carried out at 65°C in 2.5xSSC, 50 mM Na-phosphate buffer, 100 µg/ml single stranded salmon sperm DNA, and for 18 to 24 hours in a rotating hybridization oven (Robins Scientific, Sunnyvale, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden) and values were normalized against 18 S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of 18 S ribosomal subunit (Ambion Corp., Austin, TX). All analyses were performed at least three times, and all data are presented as a percentage in expression over that of the control, which were determined from parallel cultures. All data were evaluated as a mean ± 2 standard deviations with a minimum of three experiments from different populations of primary cells, and appropriate statistical analysis were performed.

RESULTS

Osteoblast-adhesion and mechanical perturbation increase levels of RGD-containing proteins:

Initial studies were carried out to assess the expression of mRNA levels for osteopontin, bone sialoprotein and fibronectin osteoblasts following either cell adhesion or mechanical perturbation. Fibronectin was used as the adhesion substrate in these experiments. The temporal profiles of *opn* expression were shown to peak at 24 hours. This induction was 3 to 4 fold above that of control samples. At time periods beyond 24 hours, there was a sharp reduction in *opn* expression, which returned to its baseline levels of expression (Figure 1). Bone sialoprotein expression followed a similar profile to that seen for *opn* also peaking at 24 hours (3 fold). However, there was no marked decrease in the expression of *bsp*, which remained elevated (2 fold) at 48 and even 72 hours (Figure 2). In contrast, the expression of *fn* mRNA started at significantly higher levels when compared to the other two mRNAs (Figure 3). Expression of *fn* peaked at 8 hours from the onset of the perturbation (2 fold), and showed a sharp reduction to its baseline levels soon thereafter.

The expression of these mRNAs was then examined after the application of mechanical perturbation. As expected, mechanical perturbation of osteoblasts increased *opn* expression by 2 to 3 folds, peaking at 9 hours post-stretch (Figure 1). This clearly contrasted with adhesion, which showed a peak induction in *opn* expression at 24 hours. In the case of *fn*, mechanical perturbation also showed an increase in expression peaking at 3 and 6 hours from the onset of the perturbation (Figure 3). It is interesting to note, however, that mechanical perturbation was inhibitory for *bsp* expression. In Figure 2, *bsp* levels started at 2 fold of control samples at 1 hour post-stretch and quickly decreased to baseline levels at 9 hours post-stretch.

These results would suggest that integrin binding-ECM molecules are selectively responsive to perturbation via either mechanical perturbation or cell adhesion mediated signal transduction. Two other prevalent ECM genes, collagen type I and osteocalcin, were then examined as a comparison to these RGD containing integrin ligands. Both collagen type I (*col1*) and osteocalcin (*oc*) mRNAs were examined after mechanical perturbation. Interestingly, neither one of these genes showed alterations in their expression when the cells were subjected to mechanical perturbation (Figure 4).

Different matrix proteins require different signal transduction pathways following cell adhesion:

In order to further understand if a common signal transduction processes mediated the induction of the three RGD integrin ligand genes, pharmacological inhibitors of specific signal transduction pathways were used. From the slow increase in *opn* levels following cell adhesion, it appears that this protein is involved in a secondary, down-stream event to other genomic changes. Indeed, the compound cycloheximide, a known inhibitor of *de novo* protein synthesis, blocked the induction of *opn* mRNA expression following adhesion to fibronectin (Figure 5). Cycloheximide also inhibited the expression levels of *fn* and *bsp* genes (Figure 6 and 7), showing that these genes were also dependent on new protein synthesis following cellular adhesion. Even though the maximum levels of *fn* occurred at 9

hours post cellular adhesion, this finding was consistent with the relative long period for maximum induction of *opn* and *bsp* (Figures 1, 2 and 3).

Previous observations had shown that changes in *opn* mRNA expression in response to mechanical perturbation were dependent on the integrity of the microfilament structure of the cell [Toma et al., 1997]. This finding is consistent to the results seen in this study (Figure 5). The role of the cytoskeleton in the signal transduction pathways for each of these mRNAs was examined in these studies. Incubation of the osteoblast cultures with the microtubule depolymerizing agent colchicine did not affect the expression of any of the mRNAs (Figures 5, 6 and 7). However, cultures treated with cytochalasin-D, a microfilament disruption agent, inhibited the levels of *opn* mRNA below those of control levels (Figure 5), following mechanical perturbation. This change was not seen for either *bsp* or *fn* mRNAs (Figures 6 and 7).

Finally, specific inhibitors for second messenger systems were used. The use of genistein, a potent inhibitor of tyrosine kinase phosphorylation, was shown to significantly inhibit the expression of *opn*, *bsp* and *fn* mRNAs. As shown in Figure 5, this finding has also been observed previously for *opn* expression in mechanically stimulated cells [Toma et al., 1997; Carvalho et al., 1997]. Genistein treatment in cells subjected to mechanical perturbation also inhibited the levels of *fn* and *bsp* mRNAs (Figures 6 and 7). The pharmacological inhibitor of PKA-like kinases, H-89, also caused an inhibition of *fn* levels, but it did not effect the expression of *opn* or *bsp* following cell adhesion. However, when H-89 was given to cells that had undergone mechanical perturbation, the expression of both *bsp* and *opn* mRNAs were inhibited, but not that of *fn* mRNA (Figures 5, 6 and 7). These findings suggest that the perturbation in expression of the RGD-containing extracellular proteins, which are studied here in response to cell adhesion, is distinct from that of mechanical perturbation, yet both responses appear to be uniquely dependent on the activation of specific subsets of kinases.

DISCUSSION

In previous studies we have shown that both mechanical perturbation and cell adhesion led to the induction of increased *opn* expression. While both types of stimuli were dependent on integrin receptors, each was mediated by a specific set of intracellular signals that were distinct for each type of perturbation [Carvalho et al., 1998]. The present study provides further evidence that both cellular adhesion and mechanical perturbation lead to the selective induction of multiple integrin binding proteins within osteoblasts. Furthermore, while these data suggest that there may be some common mechanisms of signal transduction that stimulate the increased expression of these genes, each of the genes was uniquely and separately regulated by both stimuli (Table 1).

In the bone extracellular matrix, RGD-containing glycoproteins including osteopontin, bone sialoprotein and fibronectin are presumed to interact with cell adhesion receptors (integrins) on the surface of the bone cells [Grzesik and Robey, 1994]. Studies suggest that that these extracellular components directly affect gene expression [Pienta et al., 1991], which takes place following mechanical perturbation [Resnick et al., 1993; Toma et al., 1997; Carvalho et al., 1998]. When considering adhesion separately from mechanical perturbation, one needs to take into account the effects of the former over the latter, as it may be speculated then that adhesion or integrin-ligation acts as a "primer" prior to any response due to the mechanical perturbation. In particular, it is interesting to note that the effects of the dynamic, spatially uniform mechanical perturbation on cells that are in the process of adhesion enhances the formation of new receptor-ligand bonds. In studies by Kuo et al. [1993], mechanical perturbation was shown to alter the kinetic regulation of cell adhesion, thus interfering with binding rate. However, once the cells are adherent, specific RGD tri-peptides inhibited both adhesion and mechanical perturbation responses [Carvalho et al., 1998]. Wilson et al. [1995] also presented evidence that RGD peptides, fibronectin and certain integrin antibodies disrupted integrin-ligand

interaction, which in turn ablated strain induced mechano-transduction responses, without disrupting adhesion of the same cells.

It has been proposed that receptors such as integrins can behave as a homeostatic system for modulating the extracellular matrix structure and organization in response to the structural needs of the cell [Werb et al., 1989; Gerstenfeld, 1999]. In this context, it is interesting to note that the mechanical forces that are applied to tissues should be the structurally deformed at their anchorage points to the extracellular matrix (ECM). These sites should be the focal points at which mechanical strains are specifically transmitted to the cells. The strains to which a cell is subjected, therefore, will be effected by both the compositional properties of the matrix and the receptors on the surfaces of the cells, which can interact with these ECM proteins. The importance of extracellular interactions for the process of mechano-sensation has been shown by Du et al. [1996]. In those experiments, it was demonstrated that isolated genes of the touch receptor neurons in *C. elegans* encoded for extracellular proteins. These authors [Du et al., 1996] further hypothesized that the extracellular matrix mediates the anchoring properties of specialized cells, enabling their mechano-sensory response.

Concerning the intercellular components of mechano-signal transduction, both Davies et al. [1993] and Ingber [1997] have suggested that the transduction of mechanical stimuli in anchorage-dependent cells is due to a combination of signal transduction processes via the cytoskeleton through integrin receptors that interact with the cytoskeleton, as well as through biochemical signals. In order for mechano-signal transduction to occur through the cytoskeleton, it is necessary that cooperative interactions occur between the three component parts of cytoskeleton; the microfilaments, intermediate filaments and microtubules. Studies of osteoblasts have also shown that the cytoskeleton changes both its structural architecture and its composition in response to mechanical perturbation [Meazzini et al., 1998]. However, signal transduction of different types of signals may not depend on the integrity of all of these structural elements. In this study, this is shown by following the selective inhibition of

microtubules and microfilaments. The results presented in Figure 5 and Table I demonstrate that *opn*-cell adhesion dependent mRNA expression was inhibited following colchicine treatment, yet adhesion was not affected after the addition of cytochalasin-D. The inverse effect was seen when the cells were mechanically stimulated [Toma et al., 1997]. In contrast, alterations in cytoskeletal architecture via pharmacological manipulation did not change the expression of *bsp* or *fn* mRNAs in response to either mechanical perturbation or cell adhesion. These latter results suggest the involvement of microfilaments in the selective regulation of some genes but not others. Indeed, recent studies have shown that the induction of COX 2 enzyme expression within osteoblasts in response to fluid flow mediated shear stress was also regulated via alteration in the cells cytoskeletal architecture [Pavalko et al., 1998]. Such results suggest that there may be specific subsets of genes that are commonly regulated and are dependent on the cytoarchitecture of the cell, yet others may not be dependant on the cytoarchitecture, even though they are regulated by cell adhesion or mechanical perturbation.

This differentiation between mechanical perturbation and adhesion appears to follow unique mechanisms. Since each extracellular matrix ligand interacts with different integrins, it may be speculated that selective interactions or mediation of the various signals occurs through specific integrin receptors. For instance, while $\alpha_v\beta_3$ deficient cell populations were not capable of migrating in response to *opn*, these same cells did migrate significantly in response to fibronectin and vitronectin [Liaw et al., 1995]. Furthermore, specific ligand interactions may mediate a variety of intracellular signals ranging from ion flux to selective G-protein kinase and/or phosphatase activation. The transduction of the mechanosignal at the cellular membrane leads to a cascade of downstream signaling events, many of which are mediated by tyrosine kinases, which in turn phosphorylate other kinases [Berk et al., 1995]. Kinases that have been associated with mechano-transduction include mitogen-activated protein kinase (MAPK). MAPK has been shown to follow mechanical perturbation in cardiac cells [Yamazaki et al., 1993] and fluid flow in endothelial cells [Tseng and Berk, 1993]. It has been suggested that such

responses are part of a multiplicity of pathways and might be grouped functionally into those that are either calcium dependent or calcium independent [Berk et al., 1995; Ishida et al., 1997]. The presence of calcium is important for the activation of a putative shear stress receptor (membrane level), which regulates a pertussis toxin-sensitive G protein-coupled K⁺ channel (SSR) [Ohno et al., 1993] and the enzyme phospholipase C [Nollert et al., 1990]. The levels of PIP₂, in turn will be regulated by rho, a small GTP-binding protein [Chong et al., 1994]. The calcium-independent pathway involves the activation of MAPK [Berk et al., 1995]; however, other calcium-independent tyrosine kinases such as src and FAK may also be involved in the shear stress transduction.

A common feature in the signal transduction processes that regulate the expression of all the integrin binding genes in response to either adhesion or mechanical perturbation was the inhibition of the induction of their expression by genistein. This suggests that a tyrosine kinase(s) is involved in the signal transduction, which stimulates the expression of all of these proteins. In contrast, PKA inhibition through H-89 treatment demonstrated a selective effect for *opn* and *bsp* in response to mechanical perturbation but not with cell adhesion (Figures 5 and 6). As for *fn*, H-89 inhibited the effects of adhesion but not those of mechanical perturbation (Table 1; Figure 7). In the experiments reported herein, there were not any changes in the non-RGD containing proteins collagen type I and osteocalcin (Figure 4). Integrin receptors have been described as potential mediators of mechanical perturbation [Ingber et al., 1994; Ishida et al., 1997]. Activation of integrins has been shown to induce the tyrosine phosphorylation of FAK at focal adhesion complexes [Schaller et al., 1994]. In addition, other proteins within these focal adhesion contacts, such as paxillin and src, will also be phosphorylated when exposed to flow [Girard and Nehem, 1993; Bull et al., 1994]. Our laboratory has shown that FAK phosphorylation was regulated by mechanical perturbation [Toma et al., 1997], also suggesting that the disruption of microtubules does not affect the expression of any gene studied following mechanical perturbation. This is an interesting finding as it relates to MAPK, as this kinase has been shown as the

earliest signal activated by flow at physiological stress [Tseng and Berk, 1993]. MAPK is also known as a microtubule-associated kinase [Sabe et al., 1994], suggesting a role in the cytoskeleton. However, as disruption of microtubules did not affect gene expression following perturbation, one may only speculate on the role of MAPK as a mechanical perturbation-dependent kinase. On the other hand, we have observed that adhesion alone in the presence of the microtubule-disrupting drug colchicine, blocked the induction of *opn* expression in particular. Thus, it is conceivable that MAPK plays a role in this mechanism, since this kinase has been shown to be activated by cell binding to fibronectin [Morino et al., 1995]. It has been suggested that activation of integrins is associated with the same signal events that occur when cells are exposed to flow [Vuori and Ruoslahti, 1993; Schwartz and Deninghoff, 1994; Beck et al., 1995]. However, further study is needed, as the complexity of such a response can not be understood if the responses of mechanical perturbation and adhesion are not taken into account individually.

Integrin-ligation is thought to stimulate the same signal events as mechanical perturbation [Berk et al., 1995]. Indeed, we have shown here that this is the case, even though the mechanisms that mediate both responses are uniquely different. If integrins are the mediators for mechano-transduction in both forms of activation, then there maybe several different integrin receptors acting in concert with other sensors that are specific to the mechanical activation. The dependency of mechanical perturbation effects on RGD-containing proteins in this study and the lack of response in either collagen type I or osteocalcin further demonstrate an active role of integrins in adhesion and perturbation. It is clear that integrins and focal contacts play important roles in mechano-transduction. It remains to be determined, however, how the mechanisms of adhesion cross talk with those of mechanical perturbation and which kinases and second signals are common in regulating downstream events prior to any activation in gene expression.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the expert technical assistance of Ms. Irina Simkina in preparation and maintenance of the osteoblast cell cultures. We thank Becton Dickinson Labware and Dow Chemical Company for their generous support of this project. R.S.C. acknowledges the support of the American Association of Orthodontists Foundation. This work was supported by a grant from the Department of Defense Bone Health and Military Readiness Program DAMD17-98-1-8510

REFERENCES

- Aarden EM, Nijweide PJ, van der Plas A, Alblas MJ, Mackie EJ, Horton MA and Helfrich MH. 1996. Adhesive properties of isolated chick osteocytes *in vitro*. *Bone* 18:305-313.
- Berk BC, Corson MA, Peterson TE and Tseng H. 1995. Protein kinases as mediators of fluid shear stress stimulated signal transduction in endothelial cells: a hypothesis for calcium dependent and calcium-independent events activated by flow. *J Biomech* 28:1439-1450.
- Bull HA, Brickell PM and Dowd PM. 1994. Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. *FEBS Let* 351:41-44.
- Burger EH, Klein-Nulled J, Veldhuijzen JP. 1992. Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* 7:S397-S401.
- Carvalho RS, Schaffer JL, Gerstenfeld LC. 1998. Osteoblast induction of osteopontin expression in response to cell attachment or mechanical perturbation shows common mediation through integrin receptors. *J Cell Biochem.* 70:376-390.
- Chen J, McKee MD, Nanci A, Sodek J. 1994. Bone sialoprotein mRNA expression and ultrastructural localization in fetal porcine calvarial bone: comparisons with osteopontin. *Histochem J* 26:67-78.
- Chong LD, Traynor-Kaplan A, Bokoch GM and Schwartz MA. 1994 The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* 79:507-513.
- Curtis ASG, McGrath M, Gasmi L. 1992 Localised application of an activating signal to a cell: experimental use of fibronectin bound to beads and the implications for mechanisms of adhesion. *J Cell Sci* 101:427-436.
- Curtis ASG. 1987. Cell activation and adhesion. *J Cell Sci* 87:609-611.

- Damsky CH, Werb Z. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* 4:772-781.
- Davies PF and Tripathi SC. 1993. Mechanical stress mechanisms and the cell: An endothelial paradigm. *Circ Res* 72:239-245.
- Denhart DT, Guo X. 1993. Osteopontin: a protein with diverse functions. *FASEB J* 17:1476-1481.
- Du H, Gu G, Williams CM and Chalfie M. 1996. Extracellular proteins needed for *C. elegans* mechanosensation. *Neuron* 16:183-194.
- Gerstenfeld LC. 1999. Osteopontin in skeletal tissue homeostasis: An emerging picture of autocrine/paracrine functions of the extracellular matrix. *J Bone Min Res* 14:850-855.
- Gerstenfeld LC, Chipman S, Kelly C, Lee DD, Landis WJ. 1988. Collagen expression, ultrastructural assembly and mineralization in cultures of chicken embryo osteoblasts. *J Cell Biol* 106:979-989.
- Gerstenfeld LC, Gotoh Y, Mckee MD, Nanci A, Landis WJ, Glimcher MJ. 1990. Expression and ultrastructural localization of the major phosphoprotein synthesized by chicken osteoblasts during *in vitro* mineralization. *Anat Rec* 228:93-103.
- Gerstenfeld LC, Uporova T, Ashkar S, Salih E, Glimcher MJ. 1995. Regulation of avian osteopontin pre- and posttranslational expression in skeletal tissues. *Annals NY Acad Sci* 270:67-82.
- Girard PR and Nehem RM. 1993. Endothelial cell signalling and cytoskeletal changes in response to shear stress. *Front Med Biol Eng* 5:121-125.
- Gotoh Y, Gerstenfeld LC, Glimcher MJ. 1990. Identification and characterization of the major chicken bone phosphoprotein. Analysis of its synthesis by cultured embryonic chick osteoblasts. *Eur J Biochem* 187:49-58.

- Gotoh Y, Salih E, Glimcher MJ, and Gerstenfeld LC. 1995. Characterization of the major non-collagenous proteins of chicken bone: identification of a novel 60 kDa non-collagenous phosphoprotein. *Biochem Biophys Res Commun.* 1995; 208:863-870.
- Grzesik W, Robey PG. 1994. Bone matrix RGD glycoproteins: Immunolocalization and interaction with primary osteoblastic bone cells in vitro. *J Bone Min Res* 9, 487-496.
- Guan J-L, Trevithick JE, Hynes RO. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul* 2:951-964.
- Hu DD, Lin EC, Kovach NL, Hoyer JR and Smith JW. 1995. A biochemical characterization of the binding of osteopontin to integrins alpha v beta 1 and alpha v beta 5. *J Biol Chem* 270:26232-26238.
- Hunter GK, Goldberg HA. 1994. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. *Biochem J* 302:175-179.
- Hunter GK, Goldberg HA. 1993. Nucleation of hydroxyapatite by bone sialoprotein. *Proc Nat Acad Sci USA* 90:8562-8565.
- Hynes RO. 1992. Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.
- Ingber DE. 1991. Integrins as mechanochemical transducers. *Curr Opin Cell Biol* 3:841-848.
- Ingber DE, Dike L, Hansen L, Karp S and Liley H. 1994. Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulated cell growth, migration, and tissue pattern during morphogenesis. *Int Rev Cytol* 150:173-224.
- Ingber DE. 1997. Tensegrity: the architectural basis of cellular mechanotransduction. *Ann Rev Physiol* 59:575-99.

- Ishida T, Takahashi M, Corson MA and Berk BC. 1997. Fluid shear stress-mediated signal transduction: how do endothelial cells transduce mechanical force into a biological response? *Ann N Y Acad Sci USA* 811:12-23.
- Juliano RL, Haskill S. 1993. Signal transduction from the extracellular matrix. *J Cell Biol* 120:77-585.
- Kuo SC, Hammer DA, Lauffenburger DA. 1977. Simulation of detachment of specifically bound particles from surfaces by shear flow. *Biophys J* 73(1):517-31.
- Lehrach H, Frischauf AM, Hanahan D, Wozney J, Fuller F, Boedtker H. 1979. Construction and characterization of pro alpha 1 collagen complementary deoxyribonucleic acid clones. *Biochem* 18:3146-52.
- Liaw L, Skinner MP, Raines EW, Ross R, Cheresch DA, Schwartz S.M, Giachelli CM. 1995. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. *J Clin Invest* 95:713-724.
- Loeser RF. 1994. Modulation of integrin-mediated attachment of chondrocytes to extracellular matrix proteins by cations, retinoic acid, and transforming growth factor beta. *Exp Cell Res* 211:17-23.
- McNeil RL, Berry J, D'Errico J, Strayhorn C, Piotrowski B and Somerman MJ. 1995. Role of two mineral-associated adhesion molecules. osteopontin and bone sialoprotein, during cementogenesis. *Conn Tiss Res* 33:1-7.
- Meazzini MC, Schaffer JL, Toma CD, Gray ML, Gerstenfeld LC. 1998. Osteoblast cytoskeletal modulation in response to mechanical strain in vitro. *J Orthop Res* 16:170-80.
- Miyauchi AJ, Alvarez JI, Greenfield EM, Teti A, Grano M, Colucci S, Zambonin-Zallone A, Ross FP, Teitelbaum SM, Cheresch D, Hruska KA. 1995. Recognition of osteopontin and related peptides

- by an $\alpha v\beta 3$ integrin stimulates immediate cell signals in osteoclasts. *J Biol Chem* 266:20369-20374.
- Moore MA, Gotoh Y, Rafidi K and Gerstenfeld LC. 1991. Characterization of a cDNA for chicken osteopontin: expression during bone development, osteoblast differentiation and tissue distribution. *Biochemistry* 30:2501-2508.
- Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y and Nojima Y. 1995. Matrix/integrin interaction activates the mitogen activated protein kinase $pp44^{erk-1}$ and $p42^{erk-2}$. *J Biol Chem* 270:269-273.
- Neugebauer BM, Moore MA, Broess M, Gerstenfeld LC, Hauschka PV. 1995. Characterization of structural sequences in the chicken osteocalcin gene: expression of osteocalcin by maturing osteoblasts and by hypertrophic chondrocytes in vitro. *J of Bone & Miner Res* 10:157-63.
- Nollert MU, Eskin SG and McIntire LV. 1990. Shear stress increases inositol trisphosphate levels in human endothelial cells. *Biochem Biophys Res Commun* 170:281-287.
- Oldberg A, Franzen A, Heinegard D, Pierschbacher M and Ruoslahti E. 1988. Identification of a one sialoprotein receptor in osteosarcoma cells. *J Biol Chem* 263:19433-19436.
- Pavalko FM, Chen NX, Turner CH, Burr DB, Atkinson S, Hsieh YF, Qiu J, Duncan RL 1998 Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. *Am J Physiol* 1998 ;275:C1591-601
- Pienta KJ, Murphy BC, getzenmber RH and Coffey DS. 1991. The effect of extracellular matrix injteraction on morphologic transformation in vitro. *Biochem Biophys Res Comm* 179:333-339.
- Puleo DA, Bizios R. 1992. Mechanisms of fibronectin-mediated attachment of osteoblasts to substrates in vitro. *Bone Min* 18:215-226.

- Reinholt FP, Hultenby K, Oldberg A, Heinegard D. 1990. Osteopontin: possible anchor of osteoblasts to bone. *Proc Nat Acad Sci USA* 87:4473-4475.
- Resnick NT, Collins W, Atkinson DT, Bonthron CF, Dewey CF and Gimbrone MA. 1993. Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear stress-responsive element. *Proc Natl Acad Sci USA* 90:4591-4595.
- Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresch DA. 1993. Interaction between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. *J Biol Chem* 5:9901-9907.
- Sabe H, Hata A, Okada M, Nakagawa H and Hanafusa H. 1994. Analysis of the binding of the src homology 2 domain of Csk to tyrosine-phosphorylated protein in the suppression and mitotic activation of c-Src. *Proc Natl Acad Sci USA* 91:3984-3988.
- Schaffer JL, Rizen M, L'Italien GJ, Megerman J, Gerstenfeld LC and Gray ML. 1994. A Device for the Application of a Dynamic Biaxially Uniform and Isotropic Strain to a Flexible Cell Culture Membrane. *J Orthop Res* 12:709-719.
- Schaffer JL, Toma CD, Meazzini MC, Gray ML, Gerstenfeld LC. 1996. Mechanical perturbation of osteopontin gene expression and its relationship to microfilament structure. In: *The Biological Mechanisms of Tooth Movement and Cranial Facial Adaptation*, pp 113-121, Ed. by Z Davidovitch and L Norton, Harvard Society for the Advancement of Orthodontics, Boston, MA.
- Schaller MD and Parsons JT. 1994. Focal adhesion kinase and associated proteins. *Curr Opin Cell Biol* 6:705-710.

Schaller MD, Hildebrand JD, Dhannon JD, Fox JW, Vines RR and Parsons JT. 1994.

Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH 2-dependent binding of pp60src. *Mol Cell Biol* 14:1680-1688.

Schwartz MA and Deninghoff K. 1994. αv integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they may play a minor role in adhesion. *J Biol Chem* 269:11,133-11,137.

Toma CD, Ashkar S, Gray ML, Schaffer JL, Gerstenfeld LC. 1997. Mechano-induction of osteopontin expression in osteoblasts: dependency of signal transduction on microfilament integrity. *J Bone Min Res* 12:1626-1636.

Tseng H and Berk BC. 1993. Fluid shear stress stimulates mitogen-activated protein kinase in bovine aortic endothelial cells. *Circulation* 88:I-184.

Vuori K and Ruoslahti E. 1993. Activation of protein kinase C precedes $\alpha 5 \beta 1$ integrin-mediated cell spreading to fibronectin. *J Biol Chem* 268:459-462.

Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J Cell Biol* 109:877-889.

Wilson E, Sudhir K, Ives H. 1995. Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrins interactions. *J Clin Invest* 96:2364-2372.

Winnard RG, Gerstenfeld LC, Toma C, Franceschi RT. 1995. Fibronectin gene expression synthesis and accumulation during in vitro differentiation of chicken osteoblasts. *J Bone Min Res* 12:1969-1977.

Yamazaki TK, Tobe E, Maemura K, Kaida T, Komuro I, Tamemoto H, Kadowaki T, Nagai R and Yazaki Y. 1993. Mechanical loading activates mitogen-activated protein kinase and S-6 peptide kinase in cultured rat cardiac myocytes. *J Biol Chem* 268:12069-12076.

- Yang R, Gotoh Y, Moore MA, Rafidi K, Gerstenfeld LC 1995 Characterization of an avian bone sialoprotein (*bsp*) cDNA. 1995. comparisons to mammalian *BSP* and identification of conserved structural domains. *J Bone & Miner Res* 10:632-40.
- Yang R, Gerstenfeld LC. 1996. Signal transduction pathways mediating parathyroid hormone perturbation of bone sialoprotein gene expression in osteoblasts. *J Biol Chem* 271:29839-29846.
- Yang R, Gerstenfeld LC. 1997. Structural analysis and characterization of tissue and hormonal responsive expression of the avian bone sialoprotein (*BSP*) gene. *J Cellular Biochem* 64:77-93.
- Yasuda T, Kondo S, Homma T and Harris RC. 1996. Regulation of extracellular matrix by mechanical stress in rat glomerular mesangial cells. *J Clin Invest* 98:1991-2000.

Table I. Comparison of signal transduction pathways for the mRNA expression of *opn*, *bsp* and *fn* between cell adhesion and mechanical stretching.

| Genes | <i>opn</i> | | <i>bsp</i> | | <i>fn</i> | |
|----------------------------------|-----------------|----------------|-----------------|----------------|-----------------|----------------|
| | Adhesion | Stretch | Adhesion | Stretch | Adhesion | Stretch |
| Time after peak expression | 24 hrs | 9 hrs | 24 hrs | 1 hr | 8 hrs | 3 hrs |
| <i>De novo</i> protein synthesis | yes | no | yes | no | yes | no |
| Tyrosine kinase-mediated | yes | yes | yes | yes | yes | yes |
| PKA-mediated | no | yes | no | yes | yes | no |
| Requires microfilaments | no | yes | no | no | no | no |
| Requires microtubules | yes | no | no | no | no | no |

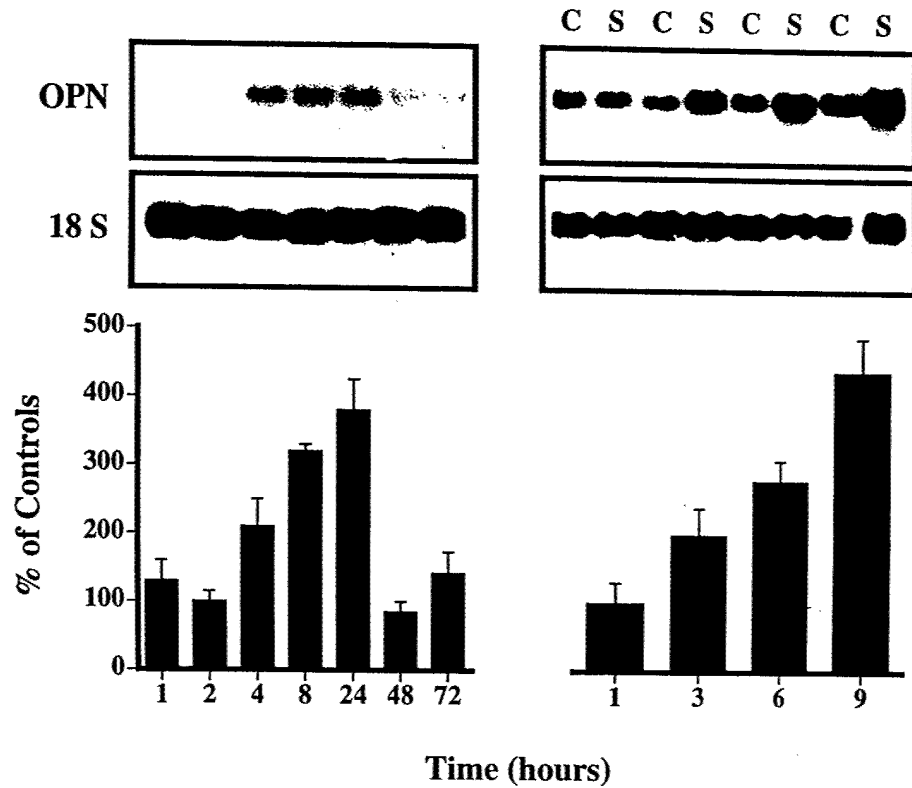


Figure 1. Effect of cell adhesion and mechanical perturbation on the temporal expression of *opn* mRNA expression by osteoblasts. Northern blot analysis of osteopontin mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical perturbation. Controls=(C) and strained samples =(S). All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments.

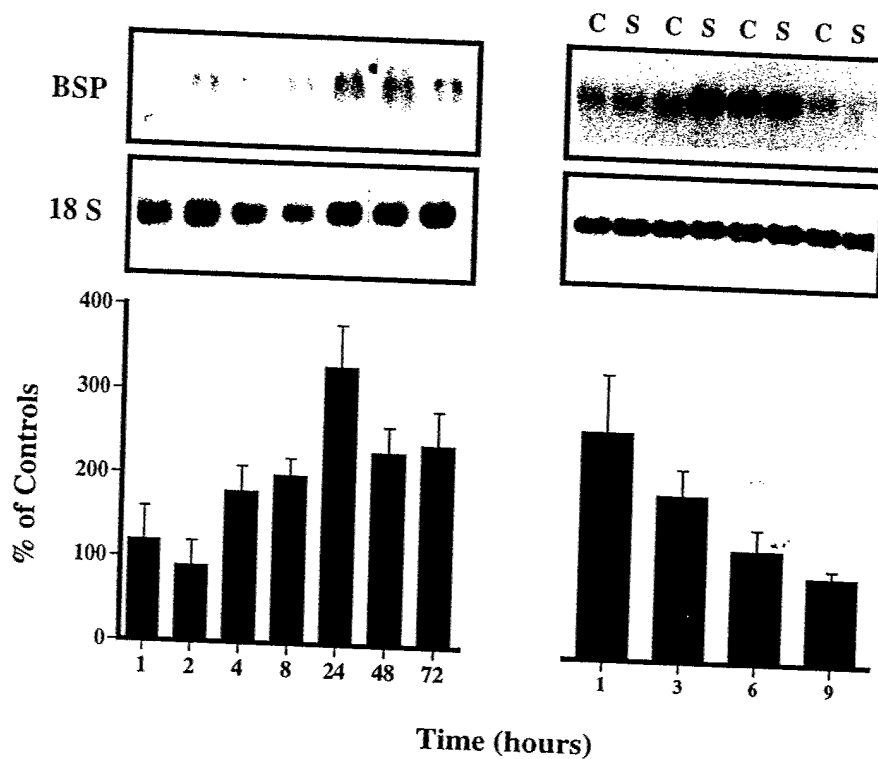


Figure 2. Effect of cell adhesion and mechanical perturbation on the temporal expression of *bsp* mRNA expression by osteoblasts. Northern blot analysis of bone sialoprotein mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical perturbation. Controls=(C) and strained samples =(S). All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments.

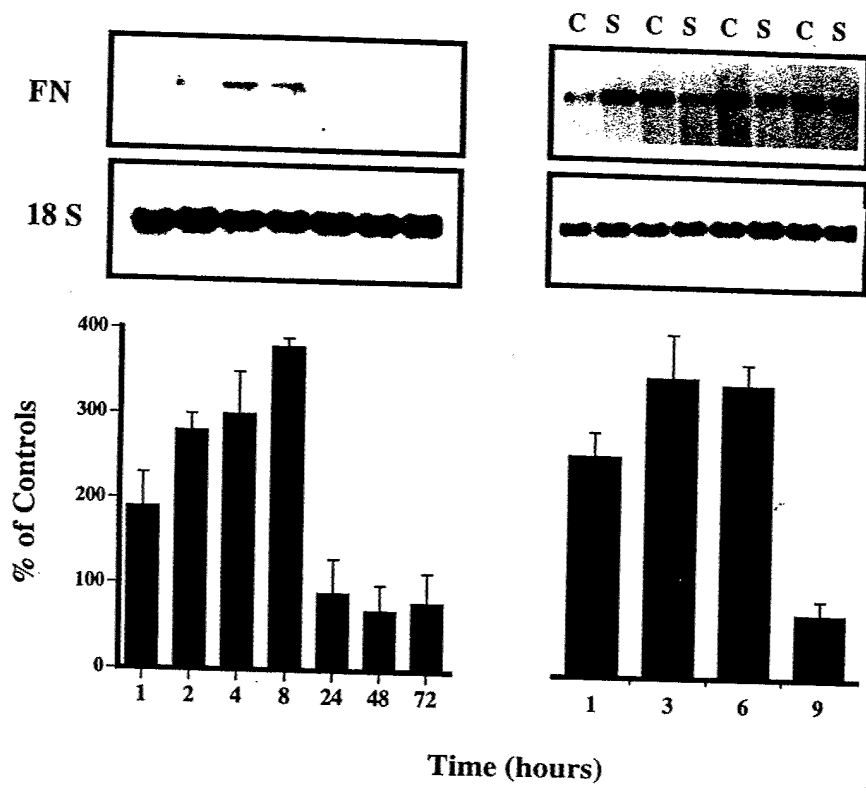


Figure 3 Effect of cell adhesion and mechanical perturbation on the temporal expression of *fn* mRNA expression by osteoblasts. Northern blot analysis of fibronectin mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical perturbation. Controls=(C) and strained samples =(S). All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments.

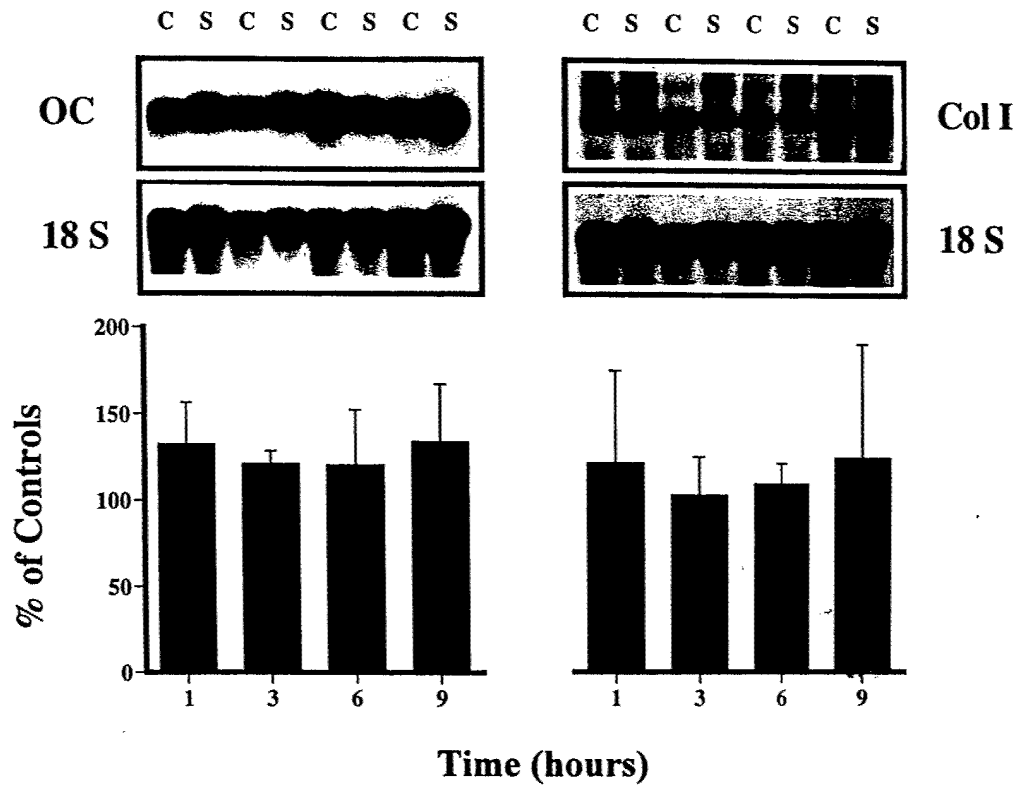


Figure 4. Effect of mechanical perturbation on the temporal expression of *coll* and *oc* mRNA expression by osteoblasts. Northern blot analysis of osteocalcin and collagen type I mRNA expression following mechanical perturbation of osteoblasts are denoted in the figure. The graphic representation of the temporal expression for these mRNAs is shown by the times of 1, 3, 6 and 9 hours post perturbation. All the panels show the percent induction of expression of the steady mRNA levels relative to their control samples. Controls=(C) and strained samples =(S). Error bars are the S.D. determined from at least three experiments.

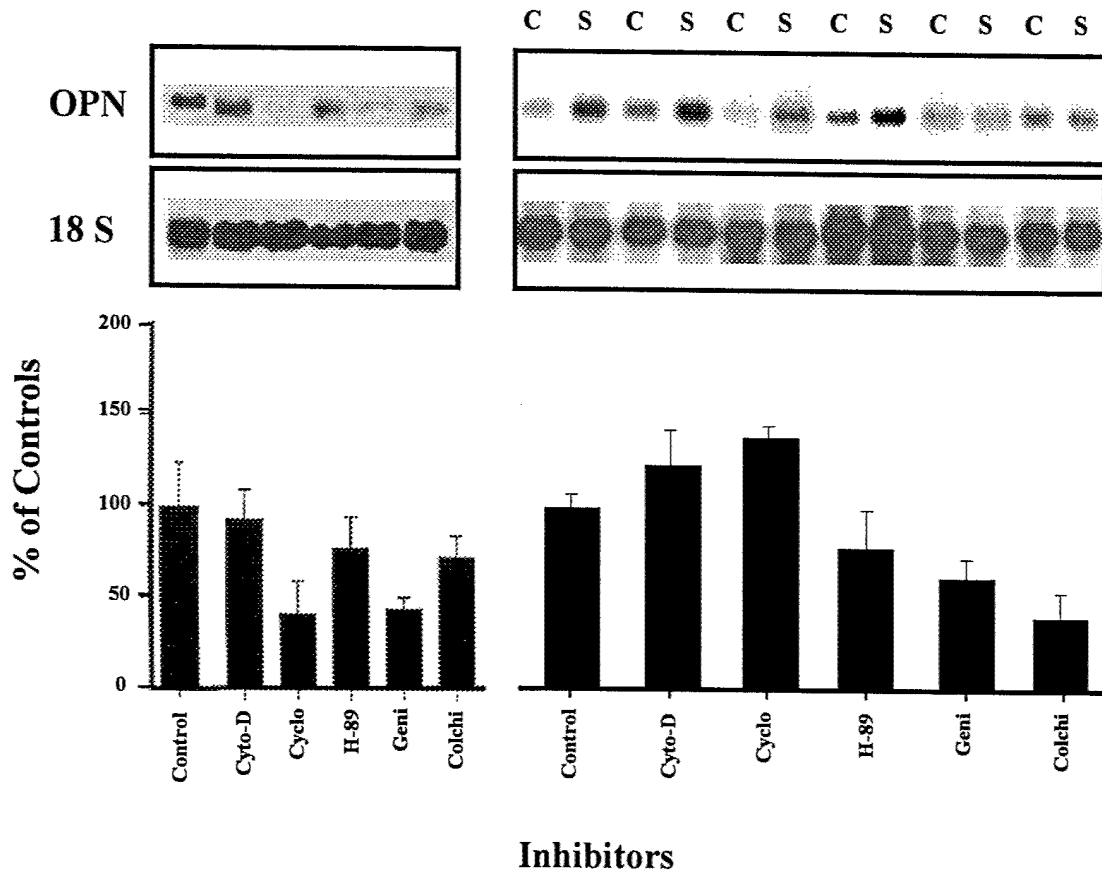


Figure 5. Effect of pharmacological inhibitors on the mRNA expression of *opn* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *opn* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolymerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of three experiments.

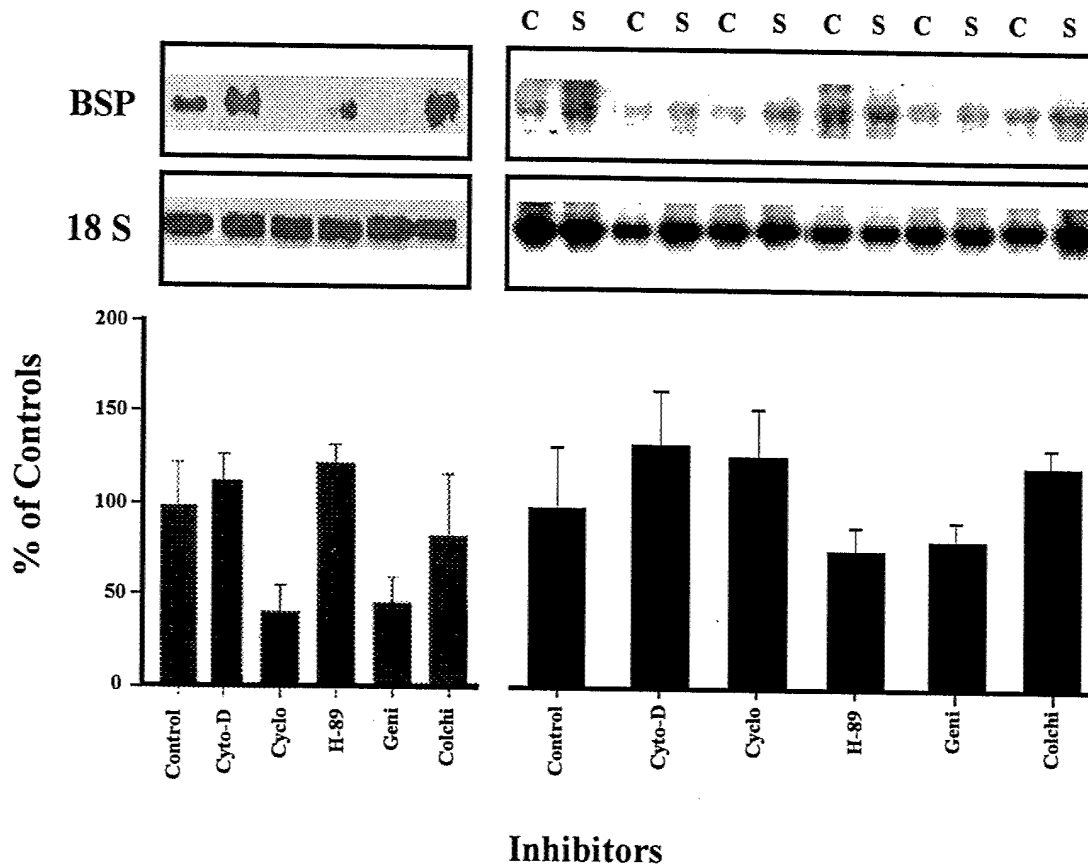


Figure 6. Effect of pharmacological inhibitors on the mRNA expression of *bsp* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *bsp* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolymerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of three experiments.

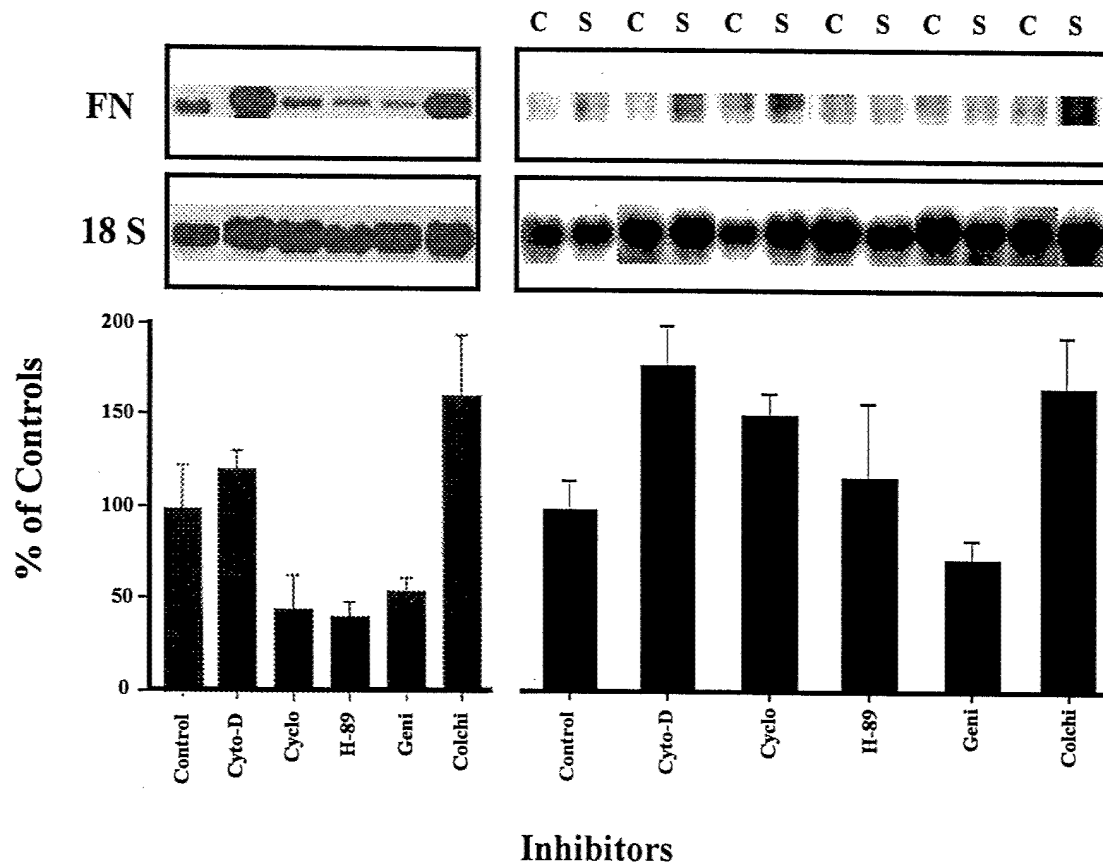


Figure 7. Effect of pharmacological inhibitors on the mRNA expression of *fn* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *fn* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolymerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of three experiments.

Appendix C

“Differential Induction of Osteopontin Gene Expression by Osteoblasts in
Response to Adhesion to Selective Integrin Ligands”

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Manuscript submitted for publication, October 2001

Final Report

DAMD17-98-1-8510

Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

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DIFFERENTIAL INDUCTION OF OSTEOPONTIN GENE EXPRESSION BY OSTEOBLASTS IN RESPONSE TO ADHESION TO SELECTIVE INTEGRIN LIGANDS

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Running title: RGD-proteins, integrins and adhesion

This work was supported by Supported by a grant from the Department of Defense Bone
Health and Military Readiness Program DAMD17-98-1-8510

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ABSTRACT: Bone formation and remodeling are partly regulated by the mechanical environment of the skeletal cells. One of the mechanisms by which mechanical stimuli may be mediated, is through cellular deformation that is transmitted through focal contact points of cell adhesion with the extracellular matrix. Previous studies have shown that osteopontin (*opn*) expression is commonly stimulated in response to both cellular adhesion. The current studies examined whether osteopontin mRNA expression in response to adhesion was mediated by specific integrin ligands. Embryonic chicken calvaria osteoblasts were plated using identical cell numbers on culture surfaces coated with fibronectin (FN) (1mg/ml), collagen type I (Col1) (1mg/ml), denatured collagen type 1 (gelatin-G) (1mg/ml), osteopontin (OPN) (1mg/ml), vitronectin (VN) (1mg/ml), laminin (LN) (1mg/ml) or poly-L-lysine (pLp) or albumin (AL). Selective adhesion was mediated by FN, COL1, G, and LN but not by pLP, AL, VN or OPN. FN generated the strongest induction of OPN expression, followed by Col1 and LN. While G was weakly inducing and neither OPN, VN, pLp or AL was capable of inducing *opn* mRNA expression. A determination of the intracellular second signaling systems (protein kinases A and C) that were activated in response to adhesion showed that both PKA and PKC activities were increased concomitantly in osteoblasts after adhesion to FN, while the other ligands that promoted cell adhesion only increased PKA activity to varying degrees. Thus, specific effects on kinase activities appear to be dependent on the selective ligand interactions, which were distinct from that of cell adhesion alone. A final set of studies evaluated the intracellular distribution of several protein components known to be within focal adhesion complexes, (FAK), and vinculin (VC) and assayed the distribution of p-Tyrosine (PT) containing proteins found within cells that were allowed to adhere either to surfaces coated either with FN or pLp. Adhesion led to the strong induction of PT with generalized change of PT containing protein

from the cell surface to the nucleus. In contrast while VC and FAK while showing increased intensity did not show any change in surface distribution. Taken together with the *opn*-mediated gene expression results, these data suggest that induction of intracellular second signal kinase activity are related to the specific nature of the ligand's interaction with the receptor and less with the process of cellular-mediated adhesion alone. They also show that the predominant integrin type that promoted both osteoblast adhesion and osteopontin gene induction was $\beta 1$ integrin and not $\beta 3$ integrin binding ligands.

INTRODUCTION

The bone remodeling cycle is known to be intimately involved in the metabolic homeostasis of mineral balance (Jilka and Manolagas, 1994). Bone formation and the remodeling cycle have been shown to be essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected (Lanyon et al., 1982; Lanyon and Rubin, 1987). Bone remodeling has also been hypothesized to provide the means of repairing bone tissue that has been damaged as a result of mechanical fatigue (Mori and Burr, 1993). Thus, it may be speculated that skeletal cells which mediate the remodeling process are regulated by their mechanical environment. It has been shown that most cell types are subjected to various forms of loading, including shear stresses due to fluid flow, stresses due to pressure, and other mechanical stimuli such as tensile stresses exerted by neighboring cells, the extracellular matrix (ECM) and the internal tension of the cytoskeleton (Shyy and Chien, 1997). Thus, in order for osteoblasts to respond to their mechanical environment, they must in some way interpret the signal generated by the stimuli. One mechanism by which cells sense these signals is through the physical deformation of the tissue (Carvalho et al., 1998). Currently, many reports have shown that signal transduction of biochemical signals following stimulation involves cell surface ligands, such as integrins (Ingber, 1991; Wang et al., 1993; Carvalho et al., 1998; Urbich et al., 2000). This is evidenced by the fact that mechanical stimulation-derived signals can only be perceived by osteoblasts and other responsive cells after cell adhesion (i.e. integrin-mediated) has taken place. Moreover, some of the signal transduction and gene expression events activated by mechanical stresses are similar, if not identical, to those induced by integrin-mediated cell adhesion (Shyy and Chien, 1997). This may be explained by changes in cell shape caused by either form of stimulation resulting in the

reorientation of the microfilament network affecting integrin behavior (Ingber, 1991). However, receptor-ligation stimulations also appear to be dependent on the positional parameters provided by the matrix. Physical alteration of ECM proteins may also change integrin conformation, facilitating the activation of specific signal transduction molecules which in turn regulate cytoskeletal arrangement and cellular response (Meazzini et al., 1997). Integrin ligands may in fact, have an autocrine and/or a paracrine function in regulating cellular function in the ECM (Gerstenfeld, 1999) and ECM proteins themselves may function as initiators of cellular activation (Carvalho et al., unpublished observations).

In bone tissue, osteopontin (OPN) is one of the predominant RGD-containing ECM proteins (Gotoh et al., 1995) and it is ubiquitously expressed in all skeletal tissues during embryogenesis (Gerstenfeld, 1999). Ultrastructural localization of OPN in areas adjacent to resorptive osteoclasts (Reinholt et al., 1990), suggests that this protein has an important role in anchoring osteoclasts to bone allowing for the resorption process. In addition, osteoclasts have been shown to synthesize OPN during active bone remodeling (Dodds et al., 1995) which further demonstrates that OPN has a major role in the maintenance of bone homeostasis (Gerstenfeld et al., 1999). We have previously shown that both mechanical stimulation (Toma et al., 1997; Carvalho et al., 1998) and cellular adhesion (Carvalho et al., 1998) result in *opn* induction, and that integrin receptors may have a common role in the signal transduction processes of both forms of stimulation. Thus, we wanted to know how osteoblasts would discriminate "ligation" at a molecular level by using different ECM "ligands" to study cell-mediated adhesion.

In order to investigate this question, it is important to understand the potential effect of selected ECM proteins in response to processes of cellular adhesion alone. Consequently, in

this study we have tested a number of ligands through attachment assays of chicken calvarial osteoblasts on tissue culture surfaces coated with fibronectin (FN), native collagen type I (Col1), denatured collagen type I (i.e. gelatin (G)), osteopontin (OPN), vitronectin (VN), laminin (LN) or polyL-Lysine (PL). Subsequently, a determination of the intracellular second signaling systems (Kinases) that are responsible for mediating cellular adhesion were determined. The selective induction of PKA and PKC occurred concomitantly only in osteoblasts from FN-coated dishes, while the other ligands promoted PKA solely. These results show that specific effects on kinase activities are dependent on the selective ligand interactions, which are distinct than those of cell adhesion alone. In addition, the strong inhibitory effect of G-coated dishes on kinase activity as measured here, indicates that biological processes that are mediated by these ligands are related to their structural conformation with their interaction with specific receptors. We also evaluated the intracellular distribution of focal adhesion kinase (FAK), phosphotyrosine (PT) and vinculin (VN) on surfaces coated with FN or PL. These results suggest that osteoblast adhesion on either surface did not appear to alter the general intracellular localization of FAK or VN. By contrast, a strong induction was noted for PT with a generalization change of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand.

Finally, we examined if integrin interactions with the same specific ligands described above were involved in the process of *opn* expression following cellular adhesion. These experiments showed that FN generated the strongest induction of *opn* expression followed by Col1 and LN. While G was weakly inducing, neither OPN nor VN was capable of inducing *opn* mRNA expression, suggesting that the latter did not facilitate specific cell adhesion. We hypothesize that other ECM proteins in addition to OPN, that are ligands for these receptors

are themselves regulated in response to cellular interaction with the matrix. Taken together with the *opn*-mediated gene expression results, these data suggest that induction of intracellular second signal kinase activity are related to the specific nature of the ligand's interactions with the receptor and less with the process of cellular-mediated adhesion alone.

MATERIALS AND METHODS

Materials

All tissue culture supplies, were from Sigma Chemical Company, St.Louis, MO. Antibodies mouse anti-chicken Vinculin, rabbit anti-human focal adhesion kinase, p125^{FAK}, mouse pTyr, goat anti-mouse FITC conjugated and sheep anti-rabbit Cy3 conjugated were also from Sigma Chemical Company, St.Louis, MO. Nylon membranes for Northern blots were from Biotrans, ICN Corp. Aurora, OH.

Cell Culture

Seventeen-day embryonic chicken calvaria osteoblasts were grown in culture as previously described (Gerstenfeld et al., 1988). These cells were plated at a density of 2×10^6 cells in 100 mm tissue culture dishes as previously described (Schaffer et al., 1994). Cultures were grown for two weeks until they reached confluence in minimum essential media supplemented with 10% fetal bovine serum (FBS). The medium was changed to BGJb supplemented with 10% FBS with the addition of 10mM β -glycerophosphate and 12.5 μ g/ml ascorbic acid. All analyses were performed on at least three separate preparations of cells and all data is presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the standard deviation (SD) of the determinations from separate experiments and the number of replicates that were used for each measurement is denoted in each figure.

Attachment Assays

For the adhesion assays the cells were initially grown one week in DME media and then subcultivated onto petri dishes that were coated with 2 $\mu\text{g}/\text{cm}^2$ or 10 $\mu\text{g}/\text{cm}^2$ of each of the proteins studied here. Fibronectin (FN), vitronectin (VN), laminin (LN), denatured collagen (i.e. gelatin) (G) and collagen type I (Coll) were obtained from Sigma Chemical Company, St Louis, MO. Osteopontin (OPN) was purified as described by Gotoh et al. (1995). FN served as the basic ligand. Dishes coated with polyL-lysine (PL) or bovine serum albumin (Al) (also from Sigma) served as controls. Different proteins were added to tissue culture plates under sterile conditions and incubated for 24 hours at 37°C. Cells were allowed to attach for one, four or twenty four hours and assays were performed at least three times for each protein ligand. Values were calculated as percentage of the cells on the experimental surfaces relative to the cell numbers observed on the plates coated with PL.

Signal Transduction Studies

Signal transduction pathways that mediate cell responses as a result of attachment/ligation were investigated measuring the specific kinase activity of the cells plated into each separate protein coating. General protein kinase C (PKC) and protein kinase A (PKA) activities were measured using specific fluorescent substrates. Enzyme activities were first normalized per total protein content used in each assay. Values were then calculated as the percentage of activities seen in cells plated on polyL-lysine coated dishes. Controls were separately determined for each compound, in cultures treated identically with the various compounds but in which the cells were attached to uncoated dishes.

Immunohistochemical studies

The intracellular distribution of focal adhesion kinase (FAK), phosphotyrosine (PT) and vinculin (VN) were examined after adhesion on surfaces coated with either PL or FN. Cell layers were washed with phosphate buffer saline (PBS) and fixed in 3.7% paraformaldehyde for 5 minutes at 4°C. Cells were then permeabilized with 0.02% triton X-100 in PBS for 10 minutes and background was blocked with blocking solution (Pierce). Cells were washed in washing buffer (0.05% casein acid hydrolysate, 0.015% Tween 20) for 10 minutes and incubated with primary antibodies (mouse anti-chicken Vinculin, rabbit anti-human FAK or mouse PT antibody for 45 minutes at 37°C. Once again cells were washed with washing buffer, incubated with secondary goat anti-mouse FITC conjugated antibodies or sheep anti-rabbit Cy3 conjugated antibodies for 30 minutes at room temperature, washed and visualized/photographed under the light microscope.

Isolation and Analysis of RNA

Total RNA was isolated using tri-Reagent™ (Molecular Center, Cincinnati, OH) according to the manufactures instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde (Toma et al., 1997) and 5 mg of total RNA was loaded per gel/lane. Chicken cDNA used for these studies was that of osteopontin (Moore et al., 1991). Northern blots with P³² cDNA-labelled probes were carried out at 65°C in 2.5 X SSC, 50 mM Na phosphate buffer, made 100 µg/ml single stranded salmon sperm DNA, and for 18 to 24 hours in a rotating hybridization oven (Robins Scientific, Sunnyvale, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden) and values were normalized to the 18 S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of 18 S ribosomal subunit (Ambion Corp., Austin, TX).

All analyses were performed at least three times and all data is presented as a percentage in expression over that of the controls determined from parallel cultures. All data were evaluated as the mean \pm 2 standard deviations with a minimum of three experiments from different populations of primary cells and appropriate statistical analysis were performed.

RESULTS

Osteoblasts have variable adhesion properties to different ligands: Initial studies were carried out to determine if chicken calvaria-osteoblasts demonstrate selective properties to different extracellular matrix proteins that serve as cell ligands. Osteoblasts were allowed to attach for four hours to FN, C, G, LN, OPN, VN or AI (used as a control). These results are seen in Figure 1, panels A and B. These data show that the different ECM proteins, FN, C and G promoted selective adhesion, while three other proteins VN, LN and OPN did not. As can be seen from these data, FN was the most effective protein in promoting selective adhesion. In addition, both natured and denatured collagen also promoted adhesion, however, these showed saturation at between 1 and 3 $\mu\text{g}/\text{cm}^2$ of surface coating. In contrast, FN did not show saturation in promoting cell adhesion until about 30 $\mu\text{g}/\text{cm}^2$ (data not shown). These results demonstrate that adhesion of osteoblasts is specifically promoted by different types of integrin ligands. Due to the response of FN in promoting osteoblast adhesion, this protein was selected as the basic cell ligand for the immunohistochemical studies described below.

Selective induction of kinase activity is related to specific protein ligand: The relationship of cell adhesion to the selective induction of specific intracellular kinase activities was examined next. In this study, cell adhesion was carried out at a fixed concentration of coating (10 $\mu\text{g}/\text{cm}^2$), considered the best concentration in preliminary observation (data not shown).

Figure 2, Panels A and B demonstrate that the profiles of selectively mediated attachment of embryonic osteoblasts was seen after only 60 minutes, compared to the profiles in Figure 1 in which attachment had been assessed for 4 hours. The induction of two classes of kinases, protein kinase A (PKA) and protein kinase C (PKC), that are involved in second signaling were then determined in identically prepared parallel cultures. It is interesting to note that attachment to FN showed induction of both PKA and PKC activities, while Coll showed the strongest induction of PKA activity with no induction of the activity of PKC. In addition, OPN also stimulated PKA activity contrasting to G and LN, which strongly inhibited PKA activity. These results show that specific effects on second signaling kinase activities are dependent on the specific ligand interactions and not on the processes of cell adhesion (Figures 1 and 2). Indeed, the strongest inhibitory effect of G on both PKA and PKC activities while at the same time promoting cell adhesion suggests that the biological processes that are mediated by these proteins are related to the structural conformation of these ligands and their interactions with specific receptors.

Fibronectin stimulates the distribution of phosphotyrosine following osteoblast adhesion:

Since FN was shown to be the most effective attachment ligand, this protein was selected to mediate cell adhesion on the distribution of three structural and enzymatic components found with focal adhesion complexes. Figure 3 shows the intracellular distribution of focal adhesion kinase (FAK), phosphotyrosine (PT) and vinculin (VN) on surfaces coated with FN or polyL-lysine (PL). These data showed that adhesion to surfaces coated with either molecule did not appear to alter the general intracellular localization or distribution of FAK nor VN within an one-hour period. On the other hand, there was a strong induction of PT levels and a generalization of its distribution throughout the cells after adhesion was promoted with a

specific binding ligand as seen in FN coating. These data reinforce the conclusion reached at Figure 2 that suggests that the induction of intracellular kinase activities are related to the specific nature of the ligand's interactions with its receptor and less with the process of cellular adhesion alone.

Attachment of osteoblasts to different ECM proteins selectively increase the expression of *opn*: In this study we examined if cell adhesion to various integrin ligands would regulate the expression of the bone-specific protein OPN. These studies also examined if integrin interactions with specific ligands were involved in these processes. The results are depicted in Figure 4. As can be seen in this figure, induction of *opn* mRNA was seen as early as 4 hours after the cells had been allowed to adhere and continued to increase up to 24 hours. At 4 hours of cell attachment, only coatings of FN and C resulted in an increase in *opn* mRNA expression. At the second time point of 24 hours, it is interesting to note that once again coating with C, FN and to a lesser extent LN generated an increase in *opn* mRNA expression. These induction were significant with a 4 fold increase following osteoblast attachment to FN and almost 3 fold increase following osteoblast attachment to C. By contrast, results showed that G, OPN and VN did not increase *opn* mRNA, therefore, these did not appear to facilitate specific cell adhesion. Taken together, these results suggest that receptor binding to different ECM proteins constitutes cellular attachment and that these selectively induce expression of *opn*.

DISCUSSION

The experiments presented here define how osteoblasts discriminate ECM-mediated signals at a molecular level. In this study we have investigated whether specific cell surface receptors that interact with ECM matrix proteins (i.e. native collagen, osteopontin, fibronectin,

denatured collagen, vitronectin and laminin) use common mechanisms in the regulation of the cellular response to adhesion.

ECM is an important structural scaffold for the organization of living tissues in the body, thus providing an array of information for several key cellular functions, such as cell motility, polarity, migration, proliferation (Damsky and Werb, 1992; Schwartz et al., 1995; Damsky, 1999) and survival (Frisch and Francis, 1994; Damsky, 1999; Urbich et al., 2000), thus preventing apoptosis (Zhang et al., 1995; Globus et al., 1998). These functions require specialized ECM proteins for a number of cell types including osteoblasts. The resultant process of osteoblast-ECM adhesion generates an abundance of signals designed to modulate vital osteoblastic responses. Transduction of signals from the ECM takes place through a variety of transmembrane proteins, including the integrin family of cell receptors (Clark and Brugge, 1995; Schwartz et al., 1995 for a review). Integrins are heterodimers comprised of two different subunits called α and β , each one of which numbering over 20 different types (Ingber, 1991). Consequently, the specificity of integrin binding will be dependent on the unique combination of these subunits (i.e. integrin $\alpha_5\beta_1$ binds fibronectin, while $\alpha_2\beta_1$ binds collagen) (Ingber, 1997).

In order to investigate cell-ECM adhesion, a number of integrin-binding proteins were selected. For instance, we selected OPN and FN since these have been shown to interact with integrins in the cell surface of bone, and are RGD-containing glycoproteins (Grzesik and Robey, 1994). In addition to the FN integrin receptor $\alpha_5\beta_1$, VN was also chosen as a ligand due the central role of its receptor ($\alpha_v\beta_3$) in cell migration and the support of cell survival (Aplin et al., 1999; Urbich et al., 2000). Similarly, LN was selected since this protein was also shown to prevent ECM-dependent apoptosis in mature osteoblasts (Globus et al., 1997).

The results presented here demonstrate that FN, C and G promoted osteoblast adhesion, while three other proteins VN, LN and OPN did not, with FN being the most effective protein in promoting selective adhesion (Figure 1). This is consistent with previous studies that also showed preferential adhesion of osteoblasts to FN and low affinity to LN (Gronthos et al., 1997). Overall, adhesion of osteoblasts appeared to be transduced primarily through integrin receptors. While, these results indicate that osteoblast adhesion is specifically promoted by β_1 and not by β_3 containing integrins, these are suggestive of a further discrimination between the individual integrin ligands since LN did not strongly mediate cell adhesion. By contrast, studies of osteoblast-like cells adhering to biomimetic peptide surfaces during shorter time periods (i.e. 30 minutes) than the ones described here (i.e. one hour >) show that in addition to β_1 , β_3 integrin may also govern cell attachment to RGD-coated surfaces (Rezania and Healy, 1999). It is not known whether shorter time points would yield similar results in our model, however, longer time points (i.e. 4 hours) in our study clearly indicated that β_1 was the primary integrin ligand (Figure 1). Figure 1 also shows that coating of C and G promoted adhesion, however, these showed saturation at between 1 and 3 $\mu\text{g}/\text{cm}^2$ of surface coating, whereas dishes coated with FN did not result in saturation by promoting cell adhesion until about 30 $\mu\text{g}/\text{cm}^2$ (data not shown). The latter may reflect affinity of the integrin-peptide interaction changes due to the microenvironment of the ligand as suggested by Houseman and Mrksich (2001). These authors also suggested that weaker interactions display a density-dependent enhancement of binding during cell attachment and spreading. Interestingly, when studying the cell adhesive shear strength and cell detachment surface energy, Yamamoto et al. (2000) reported that collagen and fibronectin-coated surfaces elicited the highest values respectively, when compared with non-specific bonding materials such as glass and polystyrene. While

these observations are in agreement with our results, these authors concluded that the number of binding sites is more important than the strength of each binding.

ECM protein-receptor specificity is an integral part of cellular signal transduction providing the means for initiation of second messenger signaling cascades (Schaller and Parsons, 1994, Damsky, 1999) which in turn go on to regulate cellular function. As a result, we subsequently looked at key signaling mechanisms following osteoblast-ECM protein adhesion. Based on the relationship of the ligand-mediated adhesion with the second signal kinase activity, Figure 2A shows that kinase activities are dependent on the specific ligand interactions and not on the processes of cell attachment alone. This is evidenced by the inhibitory effect of G coating on both PKA and PKC pathways, while at the same time promoting cell adhesion. Thus, the processes of cellular adhesion most likely are mediated solely through the RGD or other small integrin binding motifs, but the down stream activation of intracellular signaling responses are specifically related to the structural conformations of the intact ligand. In Figure 2B, one can appreciate that the activity of PKA fluctuated much more than that of PKC (i.e. 2-3 fold increase in C as opposed to no changes in LN-coated dishes). While OPN did not promote cell adhesion, it did stimulate PKA activity, while G and LN both strongly inhibited PKA activity. By contrast, the activity of PKC did not appear to change very much with exception of FN-coating, which showed a 2-fold increase versus that of control levels. These results on the second signaling kinase activities prompted us to further investigate the signaling pathways following basic osteoblast adhesion and FN was chosen as a basic ligand. The latter also stems from the fact that FN appeared to have elicited the best response in terms of cell attachment (Figure 1). In addition, it has been shown that osteoblasts

strongly depend on FN for survival once these start to form bone nodules (Globus et al., 1998), suggesting that FN may be a regulator of apoptosis in osteoblasts.

The response of osteoblast adhesion in its dependency to the ECM substrate onto which the cell adheres seen here has similar aspects to cell responses such as those elicited by mechanical stimulation (Wilson et al., 1995; Carvalho et al., 1998). Our laboratory and others have demonstrated that physical deformation of attachment substrata markedly affects protein distribution, which may or may not be transient (Meazinni et al., 1998 for a review). It is also known that ECM-derived signals are transduced to the cells through transmembrane molecules (i.e. integrins) primarily in zones of focal adhesions, which are known areas in which signal transduction takes place (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996). Therefore, signaling cascades were further investigated by the immunolabeling of two well-known focal adhesion molecules, focal adhesion kinase (FAK) and vinculin (VN) and of phosphotyrosine (PT). Although, FAK is a protein that is commonly associated with zones of focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996, Kano et al., 1999), osteoblast adhesion to FN did not appear to change its distribution (Figure 3). This result is contrasting evidence between the distinction of signal transduction of cellular adhesion to those of derived from mechanical stimulation. In the latter, FAK molecules localize to plaque-like areas of a $\alpha_v\beta_3$ integrins (Wosniak et al., 2000). It has also been shown that FAK autophosphorylation may be directly related to cell binding to OPN (Liu et al., 1997), which is consistent with the role of OPN in anchoring functional osteoclasts during bone remodeling (Reiholt et al., 1990). The role of FAK appears, however, to be highly relevant in ECM-mediated signal transduction. It has been shown that FAK transduces survival signals from ECM and that these are monitored by p53 (Ilic et al., 1998). It may be that lack of FAK redistribution following

adhesion reflects the early stage of the embryonic osteoblast phenotype studied here. In addition, PKC may also be activated by the interruption of survival signals from the ECM in conjunction with p53 (Damsky, 1999). The latter infer a close relationship between FN attachment and osteoblast survival, which is consistent with our results that showed the highest PKC activity response for dishes coated with FN as opposed to all the other ligands (Figure 2B). Since protein tyrosine phosphorylation occurs very often during intracellular signaling, the levels of phosphotyrosine were examined. Anti-phosphotyrosine staining was detected throughout the cells (Figure 3), however, it appeared mostly concentrated over areas of focal adhesions (not shown) suggesting more active events in these areas. Immediately following cell adhesion to FN, there was a strong induction of PT levels and a generalization of its distribution throughout the cells. It is speculated that this response may be directly related to β_1 integrin-mediated binding, such as the one seen when osteoblasts were subjected to mechanical stress in vitro (Bierbaum and Notbohm, 1998).

Finally, we investigated the molecular activation of intracellular signaling by looking at the osteoblast-ECM adhesion-mediated signals through their regulatory expression of *opn*, the predominant RGD-containing protein in bone (Gotoh et al., 1995). It is known that *opn* expression is regulated by both adhesion and mechanical stimulation (Toma et al., 1997; Carvalho et al., 1998). In these studies, the induction of *opn* expression at 24 hours after the cells were plated on tissue culture plastic alone or coated with these various extracellular matrix molecules. The strongest induction was seen with FN, C and LN while denatured collagen was weakly inducing and neither G, OPN nor VN was capable of inducing *opn* mRNA expression (Figure 4). These results suggest that the same integrin receptors that facilitate specific cell attachment also facilitate the induction of *opn* expression. It is

interesting to note that neither OPN nor VN mediate either of these cellular responses suggesting that the latter are not facilitated through a $\alpha_v\beta_3$ receptor, corroborating the adhesion assay results shown in Figure 1. The one difference that was observed, however, was in the comparison of cell attachment on G vs. the induction of *opn* expression. This result may suggest that signal transduction through the collagen receptor is only mediated when it interacts with native collagen. Other ligands not studied here may also offer clues as to the cell-matrix interaction following adhesion-activated signals. Byzova et al. (2000) showed that activation of $\alpha_v\beta_3$ receptor enhanced adhesive and migratory responses of human endothelial vein cells to bone sialoprotein, an important RGD-containing glycoprotein of bone cells (Grzesik and Robey, 1994). It has been suggested that certain ECM ligands (i.e. osteopontin) seem to act as autocrine factors that modify not only cell behavior in response to changes in matrix composition (Gerstenfeld, 1999) but also those changes seen following mechanical deformation (Carvalho et al., unpublished observations) by having a structural role in the matrix itself. The maintenance of the compositional balance of these various proteins in the ECM appears to maintain tissue homeostasis in response to its structural needs. In other words, the spatial and temporal assembly of ECM ligands constitutes the informational content of these molecules and characterizes the induction of cellular response following adhesion and potentially other key biological processes.

Throughout this paper, we have speculated that integrins are the mediators of ECM-osteoblast attachment, even though we have not looked directly at integrin receptors or their expression. However, the conclusion that integrins are transducing signals from the ECM is consistent with many observations published in the literature (Clark and Brugge, 1995; Gronthos et al., 1997; Liu et al., 1997; Carvalho et al., 1998; Damsky, 1999; Yamada and

Geiger, 2000; Zimmerman et al., 2000). Specifically, we have stated that β_1 was the primary integrin ligand (Figure 1). This conclusion is supported by recent experiments that showed transgenic cells expressing a dominant-negative truncated integrin β_1 subunit driven by an osteocalcin promoter in order to target its expression to mature osteoblasts and osteocytes (Zimmerman et al., 2000). In the latter studies, while wild type cells promoted osteoblast differentiation by high levels of osteocalcin and alkaline phosphatase activity, the majority of transgenic cells lost attachment and died, which further suggests that β_1 integrin is very important in the regulation of cellular adhesiveness. It is important to point out, however, that our study deals only with a small number of signaling pathways in view of the number of those describing ECM-induced integrin-dependent signaling that have been previously reported. Some of these include mitogen-activated protein kinase activation, Ca^{++} influx, pH alterations and inositol phosphate turnover (Yamada and Geiger, 1997). The investigation of these may lead to additional clues as to the regulatory influences of ECM attachment proteins.

In conclusion, adhesion of osteoblasts to ECM proteins appears to be specifically mediated by the β_1 class of integrins, in particular that of adhesion to FN through $\alpha_5\beta_1$. This same class of integrins appears to be responsible for the signal transduction process that stimulates *opn* induction. The β_3 integrin containing-ligands vitronectin and osteopontin neither mediate specific adhesion nor induce *opn* gene expression. Therefore, even though cellular attachment may occur through non-specific binding, down stream activation of intracellular signaling responses that are mediated by ECM ligands are specifically related to the ligand's structural conformation and its interactions with specific receptors.

REFERENCES

- Aplin AE, Short SM and Juliano RL. Anchorage-dependent regulation of the mitogen-activated protein kinase cascade by growth factors is supported by a variety of integrins α chains. *J Biol Chem* 399:601-605, 1999.
- Bierbaum S and Notbohm H. Tyrosine phosphorylation of 40 kDa proteins in osteoblastic cells after mechanical stimulation of beta1-integrins. *Eur J Cell Biol* 77:60-67, 1998.
- Burridge K and Chrzanowska-Wodnicka M. Focal adhesions, contractility and signaling. *Annu Rev Cell Dev Biol* 12:463-519, 1996.
- Byzova TV, Kim W, Midura RJ, Plow EF. Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. *Exp Cell Res* 254:299-308, 2000.
- Carvalho RS, Bumann A, Schaffer JL, and Gerstenfeld LC. Osteoblast induce osteopontin expression in response to attachment on fibronectin: demonstration of a common role for integrin receptors in the signal transduction processes of cell attachment and mechanical stimulation. *J Cell Biochem* 70:376-390, 1998.
- Carvalho RS, Bumann A, Schaffer JL, and Gerstenfeld LC. Integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation. *J Cell Biochem* (in press).
- Clark EA and Brugge JS. Integrins and signal transductions pathways: the road taken. *Science* 268:233-239, 1995.

- Damsky CH and Werb Z. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* 4:772-781, 1992.
- Damsky CH. Extracellular matrix-integrin interactions in osteoblast function and tissue remodeling. *Bone* 25:95-96, 1999.
- Dexter SJ, Pearson RG, Davies MC, Camara M and Shakesheff KM. A comparison of the adhesion of mammalian cells and *Staphylococcus epidermidis* on fibronectin-modified polymer surfaces. *J Biomed Mater Res* 56:222-227, 2001.
- Dodds RA, Connor JR, James IE, Rykaczewski KL, Appelbaum E, Dul E and Gowen M. Human osteoclast, not osteoblasts, deposit osteopontin onto resorption surfaces: an in vitro and ex vivo study of remodeling bone. *J Bone Min Res* 10:1666-1680, 1995.
- Frisch SM and Francis H. Disruption of epithelial cell-matrix interactions induce apoptosis. *J Cell Biol* 124:619-626, 1994.
- Gerstenfeld LC, Chipman S, Kelly C, Lee DD, Landis WJ. Collagen expression, ultrastructural assembly and mineralization in cultures of chicken embryo osteoblasts. *J Cell Biol* 106:979-989, 1988.
- Gerstenfeld LC, Feng M, Gotoh Y and Glimcher MJ. Selective extractability of noncollagenous proteins from chicken bone. *Calcif Tiss Int* 55(3):230-235, 1994.
- Gerstenfeld LC. Osteopontin in skeletal tissue homeostasis: an emerging picture of the autocrine/paracrine functions of the extracellular matrix. *J. Bone and Min Res* 14:850-855, 1999.
- Globus RK, Holmuhamedov E, Morey-Holton E and Moursi AM. Laminin as an autocrine factor for the differentiation and survival of osteoblasts. *Bone* 23(Suppl): 434, 1997.

- Globus RK, Doty SB and Lull JG, Holmuhamedov E, Humphries MJ and Damsky CH. Fibronectin is a survival factor for differentiated osteoblasts. *J Cell Sci* 111:1385-1393, 1998.
- Gotoh, Salih E, Glimcher ML and Gerstenfeld LC. Characterization of the major non-collagenous proteins of chicken bone: identification of a novel 60 kDa non-collagenous phosphoprotein. *Biochem Biophys Res Commun* 208:863-70, 1995.
- Gronthos S, Stewart K, Graves SE, Hay S and Simmons PJ. Integrin expression and function on human osteoblast-like cells. *J Bone Miner Res* 12:1189-1197, 1997.
- Grzesik W and Robey PG. Bone matrix RGD glycoproteins: Immunolocalization and interaction with primary osteoblastic bone cells in vitro. *J Bone Min Res* 9, 487-496, 1994.
- Houseman BT and Mrksich M. The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. *Biomaterials* 22:943-55, 2001.
- Ilic D, Almeida E, Schlaepfer DD, Dazin P, Aizawa S and Damsky CH. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 143:547-560, 1998.
- Ingber DE. Integrins as mechanotransducers. *Curr Opin Cell Biol* 3:841-848, 1991.
- Ingber DE. Tensegrity: the architectural basis of cellular mechanotransduction. *Ann Rev Physiol* 59:575-599, 1997.
- Jilka RL and Manolagas SC. The cellular and biochemical basis of bone remodeling. In *Osteoporosis* (ed. R. Marcus), Blackwell Scientific Publications, Cambridge MA. pgs 17-47, 1994.

- Kano Y, Katoh K and Fujiwara K. Lateral zone of cell-cell adhesion as the major fluid shear stress-related signal transduction site. *Circ Res* 86:425-433, 2000.
- Lanyon LE and Rubin CT. Functional strain on bone tissue as an objective and controlling stimulus for adaptive bone remodeling. *J Biomech* 15:141-154, 1987.
- Lanyon LE, Goodship AE, Pye C and McFie H. Mechanically adaptive bone remodeling. *Biomechanics* 17: 897-906, 1982.
- Liu YK, Uemura T, Nemoto A, Yabe T, Fujii N, Ushida T and Tateishi T. Osteopontin involvement in integrin-mediated cell signaling and regulation of expression of alkaline phosphatase during early differentiation of UMR cells. *FEBS Lett* 420:112-116, 1997.
- Meazzini MC, Schaffer JS, Toma CD, Gray ML and Gerstenfeld LC. Osteoblast cytoskeletal modulation in response to mechanical strain in vitro. *J Orthop Res* 16:170-180, 1998.
- Moore MA, Gotoh Y, Rafidi K and Gerstenfeld LC. Characterization of a cDNA for chicken osteopontin: expression during bone development, osteoblast differentiation and tissue distribution. *Biochemistry* 30:2501-2508, 1991.
- Mori S and Burr DB. Increased intracortical remodeling following fatigue damage. *Bone* 14:103-109, 1993.
- Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE and Duncan RL. Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. *Am J Physiol* 273:C810-, 1997.
- Reinholt FP, Hulthenby K, Oldberg A and Heinegard D. Osteopontin: as possible anchor of osteoclasts to bone. *Proc Natl Acad Sci USA* 87:4473-4475, 1990.
- Rezania A and Healy KE. Integrin subunits responsible for adhesion of human osteoblast-like cells to biomimetic peptide surfaces. *J Orthop Res* 17:615-23, 1999.

- Schaffer JL, Rizen M, L'italien GJ, Megerman J, Gerstenfeld LC and Gray ML. A Device for the Application of a Dynamic Biaxially Uniform and Isotropic Strain to a Flexible Cell Culture Membrane. *J Orthop Res* 12:709-719, 1994.
- Schaller MD and Parsons JT. Focal adhesion kinase and associated proteins. *Curr Opin Cell Biol* 6:705-710, 1994.
- Schwartz MA, Schaller MD and Ginsberg MH. Integrins: emerging paradigms of signal atrnsduction. *Annu Rev Cell Biol* 11:549-583, 1995.
- Shyy JYJ and Chien S. Role of integrins in cellular responses to stress and adhesion. *Cuur Opin Cell Biol* 9:707-713, 1997.
- Toma CD, Ashkar S, Gray ML, Schaffer JL and Gerstenfeld LC. Mechano-induction of osteopontin expression in osteoblasts: dependency of a signal transduction on microfilament integrity. *J Bone Min res* 12:1626-1636, 1997.
- Urbich C, Walter DH, Zeiher AM and Dimmeler S. Laminar shear stress upregulates integrin expression: role in endothelial cell adhesion and apoptosis. *Circ Res* 87:683-9, 2000.
- Wang N, Butler JP and Ingber DE. Mechanotransduction across the cytoskeleton. *Scince* 260:1124-1127, 1993.
- Winston E, Sudhir K and Ives HE. Mechanicals strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. *J Clin Invest* 96:2364-2372, 1995.
- Wosniak M, Fausto A, Carron CP, Meyer DM and Hruska KA. Mechanically strained cells of the osteoblast lineage organize their extarcellular matrix through unique sites of alphavbeta3-integrin expression. *J Bone Min Res* 15:1731-1745, 2000.

- Yamada KM and Geiger B. Molecular interactions in cell adhesion complexes. *Curr Opin Cell Biol* 9:76-85, 1997.
- Yamamoto A, Mishima S, Maruyama N and Sumita M. Quantitative evaluation of cell attachment to glass, polystyrene, and fibronectin- or collagen-coated polystyrene by measurement of cell adhesive shear strength force and detachment energy. *J Biomed Mat Res* 50:114-124, 2000.
- Zhang Z, Vuori K, Reed JC and Ruoslahti E. The $\alpha_5\beta_1$ integrin supports survival of cell on fibronectin and up-regulates Bcl-2 expression. *Proc Nat Acad Sci USA* 92:6161-6165, 1995.
- Zimmerman DL, Jin F, Leboy P, Hardy S and Damsky CH. Impaired bone formation in transgenic mice resulting from altered integrin function in osteoblasts. *Dev Biol* 220:2-15, 2000.

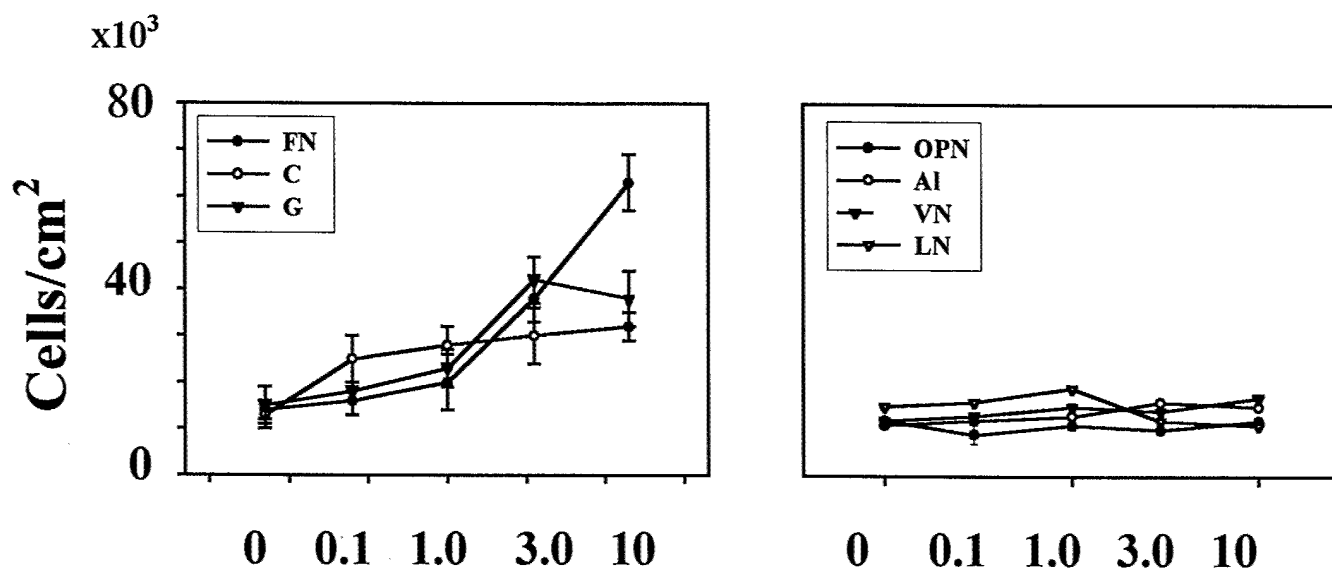
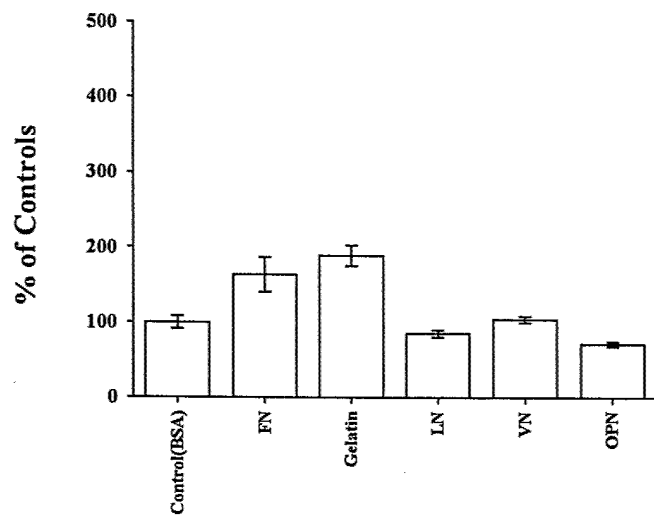
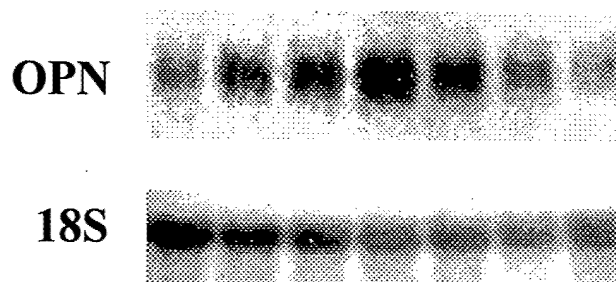


Figure 1. Relationship of ligand concentration to cell attachment. Bacterial petri dishes were coated with 0, 0.1, 1.0, 3.0 and 10 $\mu\text{g}/\text{cm}^2$ of each of the proteins. G=gelatin, denatured collagen, VN=virionectin, FN=fibronectin, OP=osteopontin, AI=bovine serum albumin, C=fibrillar collagen, native collagen, PL=polyL-lysine. Attachment was for 4 hours. Values were calculated as the total number of cells attached per cm^2 of coated surface area.

A. SPECIFIC ATTACHMENT OF OSTEOBLASTS



B. PL DN NC FN LN VN OP



C. INDUCTION OF OPN MRNA

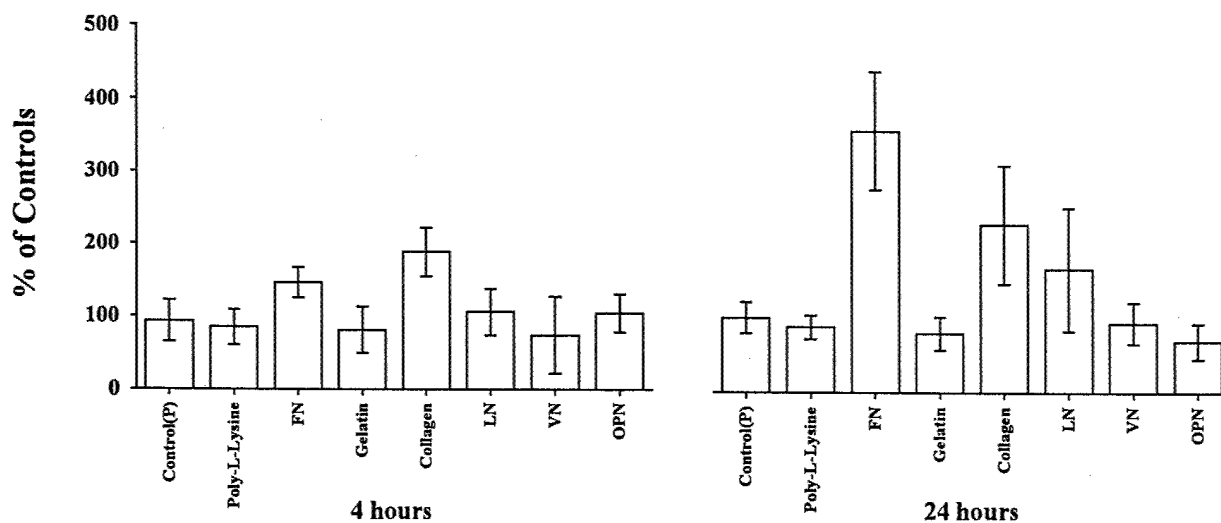


Figure 2. Comparison of selective attachment with induction of osteopontin in response to cell plating on different integrin ligands. Panel A Selective attachment of 17 day embryonic chick osteoblasts on different ligands after a four hour period. Left panel depicts the percent attachment compared to control on poly l-lysine coated surfaces after hours. All protein concentrations were at $2\mu\text{g}/\text{cm}^2$. Right panel depicts the total number of cells that were attached after four hours as a function of varying the concentration of the ligands. Panel B. Induction of osteopontin expression in response to cell attachment on various ECM proteins. Osteopontin expression was measured by Northern blot analysis and the band intensities were normalized to the 18s band(data not shown). Left panel shows percent induction after four hours and right panel after 24 hours. The nature of the protein coatings are denoted in the figure

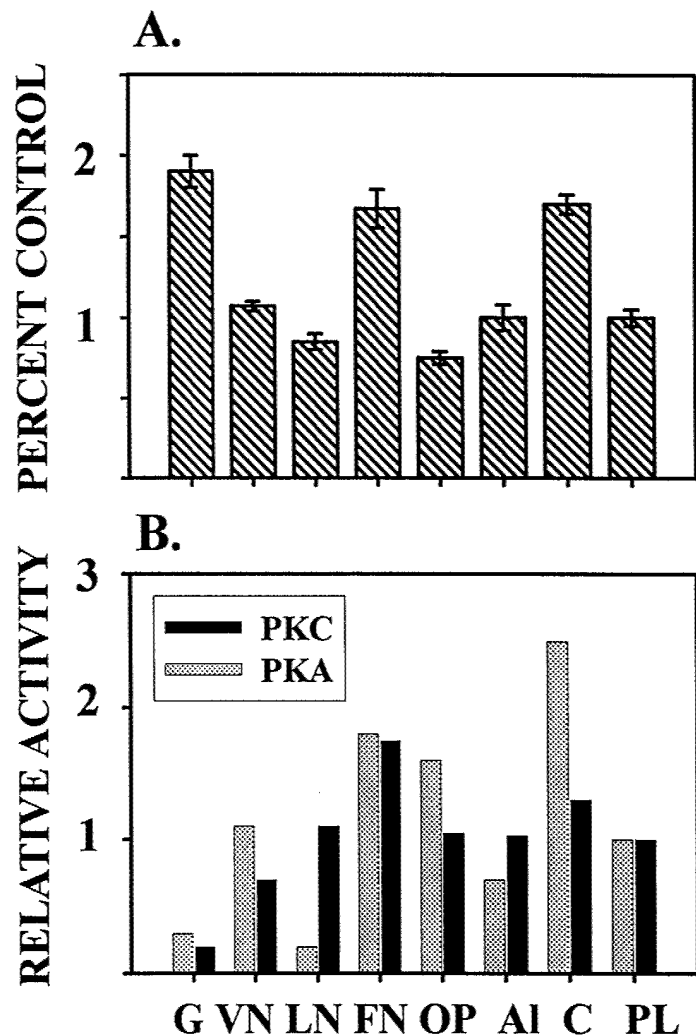
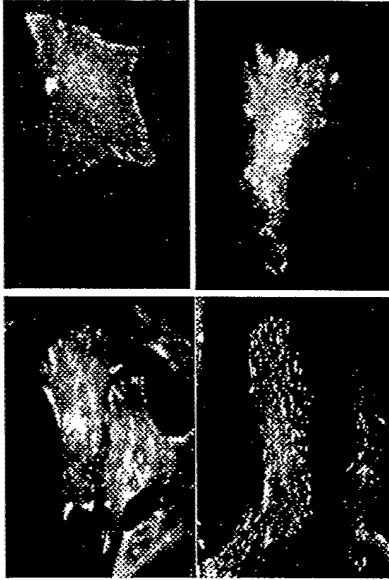


Figure 3. Relationship of Ligand Mediated Adhesion and Second Signal Kinase Activity. **Panel A.** Percent osteoblast attachment on selective protein coating. Petri dishes were coated with $2 \mu\text{g}/\text{cm}^2$ of each of the proteins. G=gelatin, denatured collagen, VN=vironectin, FN=fibronectin, OP=osteopontin, Al=albumin, C=fibrillar collagen, native collagen, PL=polyL-lysine. Attachment was for 1 hours. Values were calculated as the percentage of cells on the experimental surfaces relative to the cell numbers observed on the polyL-lysine coated dishes.

Panel B Kinase activity after adhesion on selective protein coatings. General PKA and PKC activities were measured using specific fluorescent substrates. Enzyme activities were first normalized per total protein content used in each assay. Values were then calculated as the percentage of activities seen in the cells plated on the polyL-lysine coated dishes.



Appendix D

“Induction of a Nearthrosis by Precisely Controlled Motion in an Experimental
Mid-Femoral Defect”

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Accepted for publication in the Journal of Orthopaedic Research, 2001

Final Report

DAMD17-98-1-8510

Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

P.I., Louis C. Gerstenfeld, Ph.D.



ELSEVIER

Journal of Orthopaedic Research 000 (2001) 000-000

Journal of
Orthopaedic
Research

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Induction of a neoarthrosis by precisely controlled motion in an experimental mid-femoral defect

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Received 14 February 2001; accepted 8 August 2001

Abstract

Bone regeneration during fracture healing has been demonstrated repeatedly, yet the regeneration of articular cartilage and joints has not yet been achieved. It has been recognized however that the mechanical environment during fracture healing can be correlated to the contributions of either the endochondral or intramembranous processes of bone formation, and to resultant tissue architecture. Using this information, the goal of this study was to test the hypothesis that induced motion can directly regulate osteogenic and chondrogenic tissue formation in a rat mid-femoral bone defect and thereby influence the anatomical result. Sixteen male Sprague Dawley rats (400 ± 20 g) underwent production of a mid-diaphyseal, non-critical sized 3.0 mm segmental femoral defect with rigid external fixation using a custom designed four pin fixator. One group of eight animals represented the controls and underwent surgery and constant rigid fixation. In the treatment group the custom external fixator was used to introduce daily interfragmentary bending strain in the eight treatment animals (12° angular excursion), with a hypothetical symmetrical bending load centered within the gap. The eight animals in the treatment group received motion at 1.0 Hz, for 10 min a day, with a 3 days on, 21 one day off loading protocol for the first two weeks, and 2 days on, one day off for the remaining three weeks. Data collection included histological and immunohistological identification of tissue types, and mean collagen fiber angles and angular conformity between individual fibers in superficial, intermediate, and deep zones within the cartilage. These parameters were compared between the treatment group, rat knee articular cartilage, and the control group as a structural outcome assessment. After 35 days the control animals demonstrated varying degrees of osseous union of the defect with some animals showing partial union. In every individual within the mechanical treatment group the defect completely failed to unite. Bony arcades developed in the experimental group, capping the termini of the bone segments on both sides of the defect in four out of six animals completing the study. These new structures were typically covered with cartilage, as identified by specific histological staining for Type II collagen and proteoglycans. The distribution of collagen within analogous superficial, intermediate, and deep zones of the newly formed cartilage tissue demonstrated preferred fiber angles consistent with those seen in articular cartilage. Although not resulting in complete joint development, these *nearthroses* show that the induced motion selectively controlled the formation of cartilage and bone during fracture repair, and that it can be specifically directed. They further demonstrate that the spatial organization of molecular components within the newly formed tissue, at both microanatomical and gross levels, are influenced by their local mechanical environment, confirming previous theoretical models. © 2001 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

Introduction

Joint diseases such as osteoarthritis or traumatic arthritis are characterized by the loss of articular cartilage and altered joint anatomy with resulting pain, incapac-

itation, and substantially lowered quality of life. While total joint arthroplasty is a successful treatment for these conditions, it is not a lasting solution for the young active person. Indeed, the repair of articular cartilage would be a major advance in the treatment of arthritic diseases. However, although bone regeneration during fracture healing has been demonstrated repeatedly, the regeneration of a joint structure and its articular cartilage remain elusive goals [7,8,12,24].

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50 It has long been recognized though that the me-
 51 chanical environment influences the formation of carti-
 52 lage, bone, and resultant tissue architecture during
 53 skeletal healing and in response to normal activity. The
 54 mechanical environment has been implicated in the
 55 upregulation of cartilage matrix synthesis both experi-
 56 mentally [16,30] and as a result of exercise [28]. More-
 57 over, the specific development of cartilage versus bone in

healing fractures is related to the mechanical environ- 58
 ment, a concept supported by theoretical models 59
 [2,4,6,9,11,18,27]. 60

Fracture repair involves the interaction of cells with 61
 their local mechanical environment in a cascade of 62
 molecular events, resulting in a repaired and remodeled 63
 fracture site [6,14,29]. A comparable series of interactive 64
 events also occurs in limb joint ontogeny [3,5,21,22]. 65
 Indeed, it has been demonstrated that the normal in vivo 66
 development of a joint depends on a relatively consistent 67
 mechanical load being applied [3,13,18-22]. Models of 68
 non-union have even been developed using manually 69
 applied mechanical stimulation [1]. Yet, despite the 70
 general understanding that the mechanical environment 71
 plays an important role in tissue development and re- 72
 pair, incorporation of mechanical stimulation into 73
 studies of cartilage and bone repair have been limited. 74

This study was undertaken as an empirical test of the 75
 hypothesis that precise mechanical stimulation, induced 76
 on a daily basis, can regulate osteogenic and chondro- 77
 genic tissue formation in a rat femoral defect model. Our 78
 study was designed as a confirmation of theoretical 79
 models of mechanobiological influences during skeletal 80
 healing. We demonstrate that the differentiation pro- 81
 cesses of cartilage and bone during fracture healing can 82
 be experimentally manipulated into desired tissue and 83
 structural level outcomes. This study may provide the 84
 basis for future research approaches to examine the 85
 molecular regulatory processes by which the mechanical 86
 environment regulates cartilage and bone formation, 87
 and potentially joint formation. 88

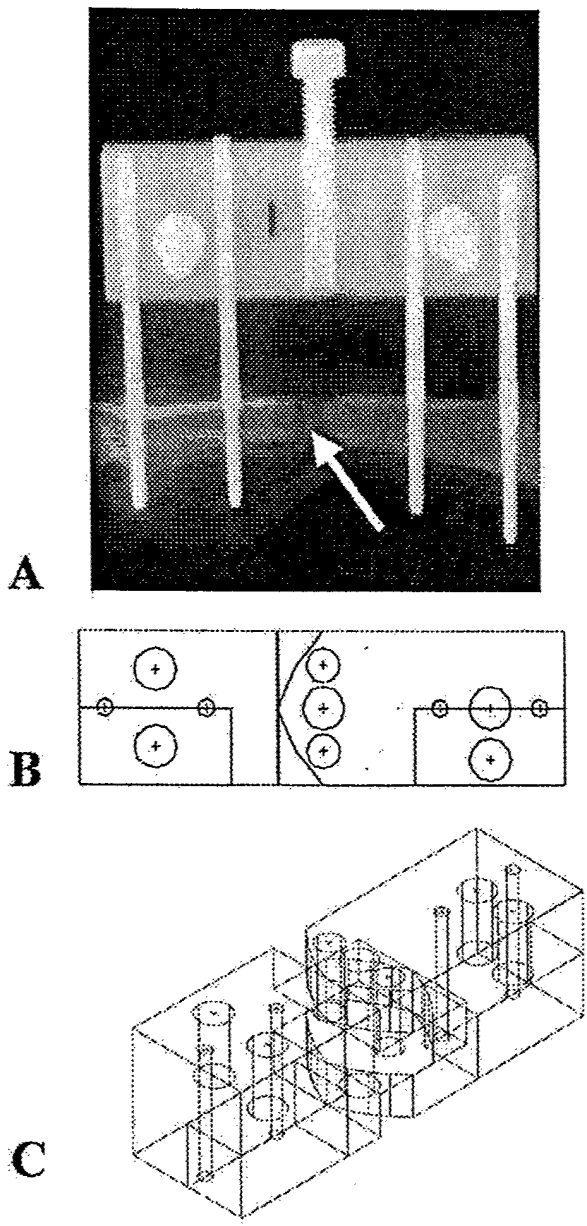


Fig. 1. Radiograph of the fixator and 3.0 mm defect on a rat femur (A) with an Autocad drawing of the fixator in lateral view (B) and obliquely in 3D (C). An arcade can be seen developing within the defect as a result of the micromotion bending treatment (arrow). The action of the fixator in (A) is in and out of the paper. In (B) the pins would be positioned in the small holes along the length of the small rectangles. Note the axis of the fixator is aligned with the theoretical center of the defect.

Materials and methods

Operative procedure

Animal care and experimental protocols were followed in accordance with NIH guidelines and approved by our institution's Laboratory Animal Science Center IACUC. Following a minimum 48 h acclimation period, sixteen skeletally mature male Sprague Dawley rats (400 ± 20 g) were transported to a dedicated surgical procedure room. Each animal was anesthetized with an isofluorene inhalation system (4.0% induction, 2.0% maintenance). The right thigh was shaved, prepped with betadine, and given an intramuscular (all injections were given via this route) injection of Cefazolin antibiotic (200 mg/kg).

A 2 cm incision was made on the lateral aspect of the thigh, creating a plane through the lateral intermuscular septum to expose the femur. The periosteum was elevated on the anterolateral aspect of the femur and a drill guide template was positioned on the femur using two cable ties. Special care was taken in placing the template to allow the pins to be inserted centrally on the diaphysis of the femur, reducing the chances of fracture due to inappropriate pin insertion. Once the template was securely placed, the proximal-most pinhole was drilled using a drill guide and a 0.7 mm drill bit. The hole was tapped with a 0.9 mm tap and the pin was secured into the femur. The other pins were placed using the same technique in order from the distal-most pin to the inner proximal and inner distal pins. The template was removed and the skin pulled over the pins by placing fourteen gauge needle heads over the pin locations. The fixator was secured to the pins as close to the skin as possible without potentially causing skin ulcerations.

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116 Following the application of the fixator, a defect was made using a
 117 rotating saw blade attached to a Dremmel Tool (Dremmel, Racine,
 118 WI). A 3 mm gap size was chosen so that it would function as a non-
 119 critical sized defect allowing healing with delayed union to occur [15].
 120 After completion of the defect, the site was irrigated with sterile saline
 121 and the fascia and skin closed. The incision was cleansed with betadine
 122 and the animal given an injection of Buprenex analgesic (0.2 mg/kg).
 123 During the 2 days following surgery each animal received an injection
 124 of Cefazolin (200 mg/kg) once per day, and an injection of Buprenex
 125 (0.2 mg/kg) twice per day. Each animal was housed in its own indi-
 126 vidual cage, and for the duration of the experiment all animals were
 127 monitored daily for pain and infection.

128 *Fixator design and application*

129 A custom designed external fixator was used to introduce 40%
 130 (vertical displacement as a function of the cortex diameter) interfrag-
 131 mentary bending strain (12° angular excursion) with a hypothetical
 132 symmetrical bending load distribution centered within the gap (Fig. 1).
 133 It was felt that 12° was well within the physiological range of angular
 134 excursion of normal developing joints such as the knee or elbow. To
 135 perform this motion an oscillating linkage was built such that the
 136 amount of interfragmentary displacement could be set between 1.0 and
 137 1.5 mm relative to the locked position, providing for the 12° of total
 138 angular excursion. The motion was applied using a stepper motor
 139 (model #2602-010, Hurst Motors, Detroit, MI). The speed of the
 140 motor was controlled using an EPC-013 stepper motor control (Hurst
 141 Motors, Detroit, MI). The shaft of the stepper motor was coupled to
 142 one of two eccentric shafts of a torque transducer (model #1102-50,
 143 Lebow Products, Troy, MI). The other eccentric shaft of the trans-
 144 ducer was connected to the linkage. A torque sensor connected to an
 145 external data acquisition board (model #100, Instrunet, Cambridge,
 146 MA) acted as a bridge voltage sensor and controlled the torque
 147 transduced to the fixator. Based on manufacturer's specifications the
 148 torque sensor was mapped to 1.967 mV/oz in., with the input torque
 149 sampled at 60 Hz for the duration of each experimental session. The
 150 peak torque required to induce motion was recorded as the baseline
 151 value. The loading apparatus was then calibrated prior to every ap-
 152 plication using Fourier transforms of the stepper motor moment ap-
 153 plication.

154 Each of the sixteen animals had an external fixator applied to its
 155 right femur and a 3 mm defect created at the mid-diaphysis. One group
 156 (*n* = 8 animals) represented the controls and remained rigidly fixed for
 157 the entire experimental period, receiving no motion treatment. The
 158 treatment group (*n* = 8 animals) received mechanical stimulation at 1.0
 159 Hz for 10 min a day, with a 3 days on, one day off loading protocol for
 160 the first two weeks. The loading protocol for the final three weeks was
 161 2 days on and 2 days off. The loading protocol was designed to give the
 162 animals weekly rest time from the anesthesia. The treatment frequency
 163 of 1.0 Hz was chosen because it falls well within the physiological
 164 range of normal joint function. A total of 600 symmetrical bending
 165 cycles per day were induced on the defect of the treatment animals for
 166 a total of 35 days (24 days of actual stimulation). The experimental
 167 time period of five weeks was determined to be the point of bony
 168 bridging under rigid fixation based upon the results of the control
 169 specimens.

170 *Radiological assessment*

171 High resolution radiographs (Ultraspeed DF-50, Kodak, Roches-
 172 ter, NY) of the harvested specimens were performed weekly until the
 173 termination of the experiment. Radiographs were taken in order to
 174 monitor fixator stability and placement, and to follow callus miner-
 175 alization and bony healing.

176 *Histological assessment*

177 Animals were euthanized at day 35, the femora were excised and
 178 specimens placed in 4.0% paraformaldehyde for 12 h. They were then
 179 decalcified for two weeks in TBD-2 decalcifying solution (Shannon,
 180 Baltimore, MD). The bones were then dehydrated via a series of in-
 181 creasing alcohol solutions and cleared in xylene for 1 h. They were
 182 infiltrated with paraffin, with the solution changed at 1 and 2 h, and
 183 maintained at 60 °C under a 25 mm Hg vacuum. The bones were

184 embedded so as to expose the sagittal plane for sectioning. Specimens
 185 were sectioned at 5-7 µm thickness and stained with hematoxylin and
 186 eosin, or alcian blue. Immunohistochemical staining for Type II col-
 187 lagen was performed using a primary antibody of Collagen II (Neo-
 188 markers, Fremont, CA) and a Vector Stain Kit (Vector Laboratories,
 189 Burlingame, CA). Articular cartilage specimens were obtained from
 190 the knees of cohort rats.

191 *Fourier transform analysis*

192 Histological slides were utilized to quantify preferred fiber angle
 193 and fiber angle conformity in the collagen fibers of the newly formed
 194 cartilage tissues (Fig. 2). These values were determined for deep, in-
 195 termediate, and superficial layers and were compared between the
 196 treatment group, the controls, and normal rat knee articular cartilage.
 197 First, standard H & E histologic sections of demineralized treatment,
 198 control, and normal rat knee cartilage specimens were prepared and
 199 collagen fiber orientation within the tissues was graphically visualized
 200 under polarized light with an Olympus OMT2 microscope at 200×
 201 magnification. Two deep, two intermediate, and two superficial fields
 202 were generated and mapped for each of the 16 cartilage surfaces (8
 203 distal and 8 proximal). The superficial zone was determined as the area

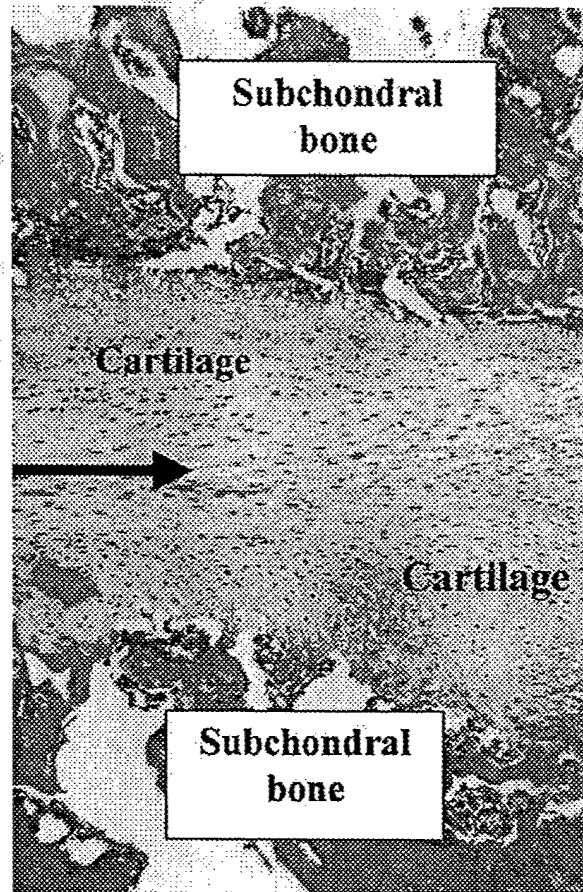


Fig. 2. Cartilage tissue formed in a *nearthrosis* via controlled micro-
 motion (from Fig. 4(D)). Red tissue at top and bottom are distal and
 proximal subchondral bone arcades. The morphology of the cartilage
 cells changes from the arcades to the midline, becoming more flattened.
 A distinctive cleavage can be seen beginning between the distal and
 proximal cartilage bands (arrow). It is believed that the introduction
 of shear micromotion would further enhance the cleavage of this cartilage
 zone into two discontinuous cartilage surfaces.

204 immediately under the cartilage surface, the deep zone was the area
 205 immediately adjacent to the underlying subchondral bone, and the
 206 intermediate zone was exactly half way between the deep and super-
 207 ficial zones. The polarized microscopic images were captured using a
 208 digital camera (Kodak DCS-420, Kodak, Rochester, NY) affixed to
 209 the microscope. The digital images were imported into Matlab and
 210 thresholds were set to obtain high contrast images (Fig. 3(A)). Preferred
 211 fiber orientation within each field was determined by creating
 212 Fourier transforms of the digitized images (Matlab, Mathworks, Nat-
 213 tick, MA), which generate an elliptical signal (Fig. 3(B)). The gray level
 214 of each pixel was processed using a 2D fast Fourier transform $F(m, n)$,
 215 where m and n are spatial frequencies corresponding to the x and y
 216 axes of the original image. The gray level of each pixel, represented by
 217 a function, $f(m, n)$, was then transformed using a 2D discrete Fourier
 218 transform.

$$F(p, q) = \sum_{m=0}^{M-1} \sum_{n=0}^{N-1} f(m, n) e^{-j(2\pi/M)pm} e^{-j(2\pi/N)qn},$$

$$p = 0, 1, \dots, M - 1, \quad q = 0, 1, \dots, N - 1.$$

220 The preferred orientation in the original image was represented by a
 221 peak in the power spectrum ($|F(m, n)|^2$, a 2D array, (Fig. 3(C))). To
 222 quantify the orientation its intensity was determined as the angular
 223 distribution of the power spectrum. The angular distribution was
 224 computed as the average of the power spectrum within a narrow fan-
 225 shaped region. The largest peak of the intensity of orientation was
 226 perpendicular to the preferred orientation of the fibers. Collagen fiber
 227 conformity was determined by the ratio of the major and minor axes of
 228 the power spectrum peak, with larger values corresponding to greater
 229 angle conformity.

230 *Statistical analyses*

231 Data are presented as mean \pm S.D. All histomorphometric results
 232 including collagen preferred fiber angle and fiber angle conformity
 233 were compared between the control and treatment groups using an
 234 ANOVA and Tukey's post hoc test at an level of 0.05, and with p
 235 values less than 0.05 interpreted as significant. All sample sizes for
 236 the specific groups were determined by power statistics calculations: based
 237 on a coefficient of variation of 25% in the data, and accepting α and β
 238 errors of 5.0%.

239 **Results**

240 All six specimens of the control group demonstrated
 241 at least partial bony union, with two showing complete
 242 union (Fig. 4). Radiological and histological analyses of
 243 the treatment group showed the creation of an orga-

nized pseudoarthrosis with opposing bone arcade
 structures capping the defect termini (Figs. 4 and 5).
 Additionally, a cartilage tissue with articular cartilage-
 like organization developed on the defect side of the
 bone arcades (Fig. 2). This complex structure was
 maintained in the mechanical stimulation group past the
 time of healing in the controls, with the shape of the
 arcades corresponding to the pivoting action of the fix-
 ator. No control animals demonstrated development of
 a bony arcade or cartilaginous band across the medul-
 lary canal. These arcades, especially when present on
 both sides (Fig. 4(B)-(D)), had the effect of segmenting
 the femur into two bones via a joint-like structure. This
nearthrosis was maintained in the treatment group past
 the time of healing in the controls. The tissue arising
 from the arcades stained positively for sulfated proteo-
 glycan specific alcian blue staining, an indication of the
 presence of cartilage. Likewise, type II collagen was
 prevalent throughout the tissue, confirming it to be
 cartilage. However, the cartilage band was not thick
 enough to quantitatively determine a trend in staining
 intensity, as is normally found in articular cartilage. The
 presence of other specific cartilage types (i.e., Type IX,
 Type X, Type XI, etc.) was not determined in this study.

The distribution and orientation of collagen fibers
 within the experimental cartilage closely resembled that
 of collagen in articular cartilage from rat knee joints,
 with an alignment parallel to the articulating surface in
 the superficial zone and an increasingly perpendicular
 alignment, relative to the articulating surface, in the
 intermediate and deeper zones (Table 1). The control
 cartilage (residual from the endochondral osteogenesis
 process of healing), was fragmented in its distribution
 within the defect and demonstrated no superficial-to-
 deep polarity in the level of organization. The greatest
 similarity in collagen fiber angle was found between the
 treatment ($4.47^\circ \pm 2.65$) and knee articular cartilage
 ($4.5^\circ \pm 4.69$) in the superficial zone ($p = 0.89$). The
 values from the control superficial zone

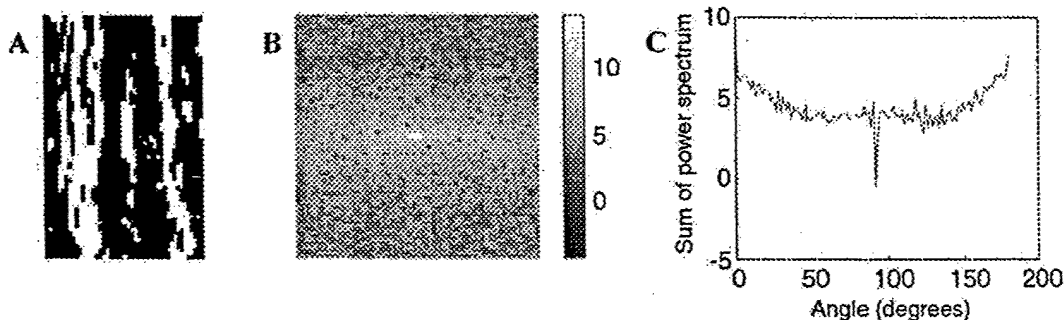


Fig. 3. Stages and results of Fourier transform of polarized nearthrotic collagen fiber images. (A) Thresholded polarized image of collagen fibers in the superficial zone of the cartilage. (B) Elliptical transform of image in 3A with horizontal major axis and (C) power spectrum analysis of fiber angle preference (90°). The ratio of the major axis to the minor axis in the ellipse in (B) determines fiber angle conformity. Higher ratios denote higher fiber conformity to preferred angle.

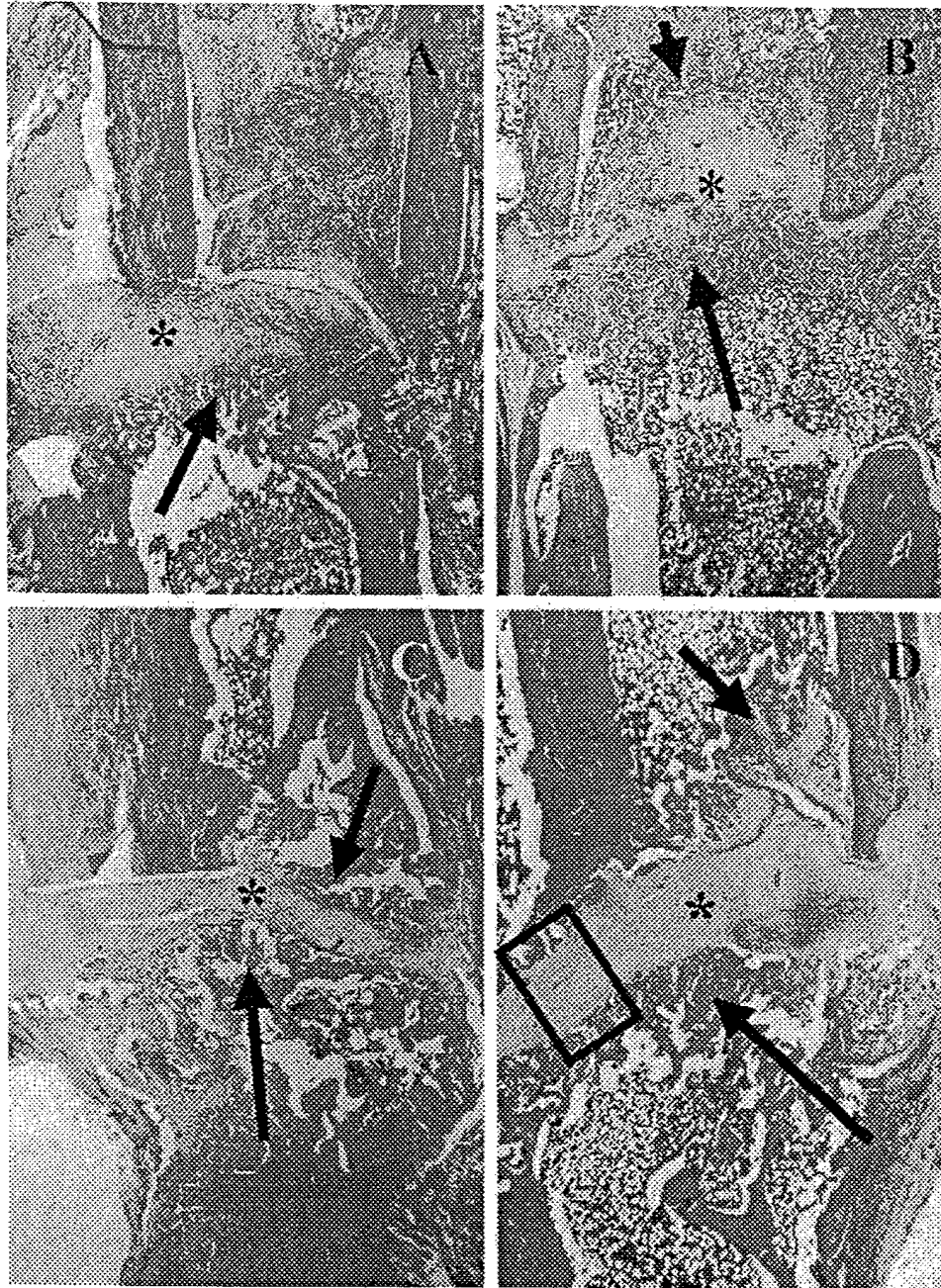


Fig. 4. Bony arcades (arrows) clearly formed in four out of six treatment animals as a result of the controlled micromotion. The bony arcade develops as an extension of the cortices and the convex formation is sometimes matched by a corresponding concave formation on the other side of the defect (B, C, D). The arcades support an articular-like cartilage tissue on the defect side (asterisk). The boxed in area (D) shows the articular-like cartilage forming on the subchondral bone, and at higher magnifications a cleavage line can be seen between distinct cartilage bands (Fig. 2).

283 (101.67° ± 105.7) were significantly different from the
 284 articular cartilage ($p < 0.01$) and treatment groups
 285 ($p = 0.04$). In the intermediate zone the knee articular
 286 cartilage and treatment groups were not significantly
 287 different (7.1° ± 4.62 and 6.55° ± 4.9, respectively,
 288 $p = 0.83$). The collagen in the intermediate zone of the
 289 control group (35.89° ± 12.72) demonstrated a some-

what perpendicular fiber angle relative to a hypothetical
 articular surface at the midpoint of the defect. There was
 a significant difference between the articular cartilage
 and the controls ($p < 0.01$), and between the neoarth-
 rotic cartilage and the controls ($p < 0.01$). The deep
 layer in all three groups demonstrated perpendicular
 preferred collagen fiber angles (relative to the articular
 surface).

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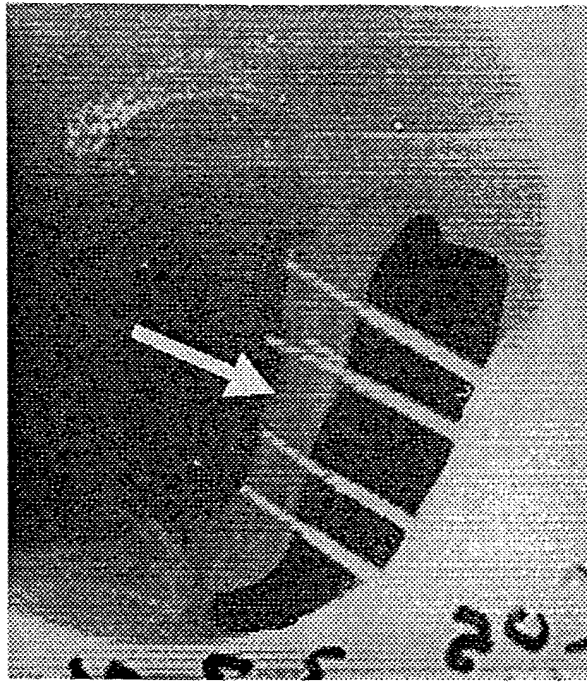


Fig. 5. Histological section of a control specimen demonstrating bony bridging of the defect via direct periosteal bone formation (arrow). Note the lack of endochondral bone formation within the defect space which is the result of ideally rigid fixation (asterisks). Two control specimens demonstrated bony healing while four showed delayed union. No control specimen developed a bony arcade or articular-like cartilage as seen in the treatment group (Fig. 4).

297 surface), with no significant differences between the
298 three groups.

299 The conformity ratios (angular agreement of the in-
300 dividual collagen fibers) were highest in the superficial
301 zones of both the knee articular cartilage and the
302 treatment cartilage (6.88 ± 3.83 and 4.04 ± 0.45 , re-
303 spectively, $p = 0.16$) while the control group demon-

strated relatively low fiber organization (1.49 ± 0.11).
However, a significant difference was found between
both the articular and nearthrotic fiber angle conform-
ities versus the control conformity ($p = 0.02$ and
 $p < 0.01$, respectively). The only significant difference in
fiber conformity in the intermediate zone was found
between the articular cartilage and the control groups
($p < 0.01$). There were no significant differences in fiber
conformity between the groups in the deep zone. In
summary, the collagen within the nearthrotic cartilage
appeared to not be statistically different from that seen
in rat knee articular cartilage, while the control cartilage
was statistically different.

Discussion

It is well known that humans are capable of forming
a cartilaginous joint-like structure in a long bone, as is
observed in the pathological condition pseudoarthrosis
[12]. Although the precise mechanical and biological
events which lead to this condition are not known,
theoretical modeling of tissue development within a
fracture site predict that such an outcome is possible
[2,4,6,9,18,27]. Experimental models of pseudoarthroses
have even been established using manually applied me-
chanical stimulation [1], but precisely controlled me-
chanical treatments and their effects on tissue
differentiation have not been established.

Our results suggest that the application of precisely
controlled bending motion in a non-critical sized defect
leads to the directed formation of specific tissues such as
cartilage (as specified by theoretical models), and that
the architectural organization of the resulting anatomical
structures relate directly to the action and magnitude
of the induced motion. Thus, the resulting pseudoarth-
rotic structures were likely a response to the symmetrical
bending motion, as are the analogous structures in in

Table 1
Results of the collagen fiber preferred orientation and conformity analyses using Fourier transforms of polarized histological specimens

| | | Superficial layer | | Intermediate layer | | Deep layer | |
|---------------------|------|----------------------|--------------------|---------------------|---------------|------------|-------|
| | | Angle (°) | Ratio | Angle (°) | Ratio | Angle (°) | Ratio |
| Articular cartilage | Mean | 4.5 α | 6.88 α | 7.1 α | 4.87 α | 64.1 | 2.97 |
| | S.D. | 4.69 | 3.83 | 4.62 | 1.34 | 31.71 | 1.39 |
| Neo arthrosis | Mean | 4.47 β | 4.04 β | 6.55 β | 3.33 | 53.2 | 1.83 |
| | S.D. | 2.65 | 0.45 | 4.9 | 1.16 | 70.09 | 0.56 |
| Healing controls | Mean | 101.67 $\alpha\beta$ | 1.49 $\alpha\beta$ | 35.89 $\alpha\beta$ | 2.16 α | 76.65 | 2.32 |
| | S.D. | 105.76 | 0.11 | 12.72 | 0.35 | 82.24 | 1.29 |

Preferred fiber angle is relative to the articulating surface except in the healing controls which were referenced to a homologous line perpendicular to the long axis of the bone and through the center of the osteotomy. The fiber angle conformity ratio is the similarity of each individual fiber angle to the average angle, with higher values indicating greater conformity. A striking similarity existed between the nearthrotic cartilage (Neo Arthrosis) and rat knee articular cartilage (Articular Cartilage) in the intermediate and superficial layers. These were in contrast to the endochondral cartilage found in typical healing fractures such as that represented by the controls. Fiber angle conformity was relatively low in the controls and the standard deviation of the mean preferred fiber angle was very high. Significant differences are denoted as (α) for articular cartilage and (β) for nearthrotic cartilage.

339 utero joint development largely a product of their me-
 340 chanical environment [3,5,13,22,23]. This cyclical and
 341 symmetrical bending motion created a mechanical en-
 342 vironment of alternating hydrostatic compression and
 343 tension in one axis, symmetrically increasing in magni-
 344 tude with increased distance from the center of the de-
 345 fect to the outer cortex. A neutral axis with respect to
 346 tension and compression was created along the theo-
 347 retical bending axis of the defect. Additionally, the ini-
 348 tiation of a line of cavitation between the newly
 349 developing surfaces resulted from the shear component
 350 of the bending action (Fig. 2, see arrow).

351 The bone arcades that formed across the termini of
 352 the defect effectively capped the segments and created
 353 what could be considered two bones out of the single
 354 femur. This segmenting of a single skeletal element is a
 355 crucial event in joint formation. The bone caps were
 356 likely the result of the forces induced by the alternating
 357 compressive loads caused by the fixator, compressing
 358 the material and cells recruited to repair the defect. In
 359 some instances clearly reciprocal convex and concave
 360 shaped arcades formed from this bone; likely a result of
 361 the pivoting action of the fixator. The arcades that de-
 362 veloped were aligned with the fixator bending axis and
 363 as the fixator pivoted on its axis so did the healing de-
 364 fect.

365 The cartilage tissue that developed on these bony
 366 caps demonstrated an organized collagen fiber archi-
 367 tecture (with respect to the perpendicular of the long
 368 axis of the bone segments). Likewise, collagen fiber ar-
 369 chitecture in articular cartilage is well organized show-
 370 ing superficial to deep polarity [10,26]. In contrast,
 371 collagen fiber architecture in cartilage of the endo-
 372 chondral ossification pathway is typically much less
 373 organized, having little to no deep-to-superficial polarity
 374 in its structure. Although not identical to articular car-
 375 tilage, the ultrastructure of the cartilage that developed
 376 on the bony termini of the experimental group demon-
 377 strated a degree of organization which was strikingly
 378 similar.

379 A shear component was also induced on the surface
 380 of the defect tissues as they developed, another result of
 381 the pivoting action imposed by the fixator. As the tissue
 382 initially began to differentiate, the plane of shearing
 383 action defined the dividing point between the segments.
 384 As the tissues further differentiated into bone and car-
 385 tilage, the shear component was likely responsible for
 386 the specialized orientation of the collagen fibers within
 387 the superficial layer of cartilage. Shear forces would be
 388 greatest superficially at the opposing surfaces (the lo-
 389 cation of greatest shear displacement during bending),
 390 and be reduced in magnitude proximally and distally
 391 from the defect center. Thus, shear would be lower in
 392 magnitude in the deeper cartilage tissues where the
 393 collagen fiber orientation was increasingly less tangen-
 394 tial to the presumptive surface, and deeper still where

subchondral bone formed. This is analogous to the
 mechanical environment responsible for articular carti-
 lage formation in utero.

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Uncited reference

[25].

Acknowledgements

The authors would like to recognize Robert Sjostrum
 for machining the fixators and linkage system. Amy
 Frederick's salary was partially funded by the NIH,
 HD22400, and Dr. Cullinane's salary was partially
 funded by the Department of Defence, DAMD 17981.
 The authors received no commercial funds for this ex-
 periment.

References

[1] Brighton CT, Krebs AG. Oxygen tension of nonunion of fractured
 femurs in the rabbit. *Surg Gynecol Obstet* 1972;135:379-85.
 [2] Carter DR. Mechanobiology of skeletal tissue growth and
 adaptation. In: *Proceedings of the 12th Conference on Europ
 Soc Biomech*, 2000. p. 7.
 [3] Carter DR, van der Meulen MCH, Beaupre GS. Mechanobiologic
 regulation of osteogenesis and athrogenesis. In: *Buckwalter JA,
 Ehrlich MG, Sandell LJ, Trippel SB, editors. Skeletal growth and
 development: clinical issues and basic science advances*. Rose-
 mont, IL: *Am Acad Orthop Surg*; 1998a. p. 99-130.
 [4] Carter DR, Beaupre GS, Giori NJ, Helms JA. Mechanobiology of
 skeletal regeneration. *Clin Orthop Suppl* 1998b;355:S41-55.
 [5] Carter DR, Wong M. Mechanical stresses in joint morphogenesis
 and maintenance. In: *Mow VC, Woo SLY, Ratcliffe A, editors*.

444 Biomechanics of diarthrodial joints, vol. 2. New York: Springer;
 445 1990. p. 155-74.

446 [6] Carter DR, Blenman PR, Beaupre GS. Correlations between
 447 mechanical stress history and tissue differentiation in initial
 448 fracture healing. *J Orthop Res* 1988;6:736-48.

449 [7] Chen FS, Chao Y, Shang Q. Advances in the research on repairing
 450 cartilagenous defects of synovial joint. *Chung Kuo Hsiu Fu*
 451 *Chung Chien Wai Ko Tsa Chih* 1998;12(5):297-300.

452 [8] Chen FS, Frenkel SR, Di Cesare PE. Repair of articular cartilage
 453 defects: part II. Treatment options. *Am J Orthop* 1999;28(2):88-
 454 96.

455 [9] Claes LE, Heigele CA. Magnitudes of local stress and strain along
 456 bony surfaces predict the course and type of fracture healing. *J*
 457 *Biomech* 1999;32:255-66.

458 [10] Cova M, Toffanin R, Frezza F, Pozzi-Mucelli M, Mlyn'arik V,
 459 Pozzi-Mucelli RS, Vittur F, Dalla-Palma L. Magnetic resonance
 460 imaging of articular cartilage: ex vivo study on normal cartilage
 461 correlated with magnetic microscopy. *Eur Radiol* 1998;8(7):1130-
 462 6.

463 [11] Cullinane DM, Inoue N, Meffert R, Tis J, Rafiee B, Chao EYS.
 464 Regulating bone at the cellular level: A little evidence for Wolff's
 465 Law. *Am Zool* 1999;39(5):96.

466 [12] Dorr LD. Continuous passive motion offers no benefit to the
 467 patient. *Orthopaedics* 1999;22(4):393.

468 [13] Drachman DB, Sokoloff L. The role of movement in embryonic
 469 joint development. *Dev Biol* 1966;14:401-20.

470 [14] Einhorn TA. The cell and molecular biology of fracture healing.
 471 *Clin Orthop* 1998;355(Suppl.):S7-21.

472 [15] Einhorn TA. Clinically applied models of bone regeneration in
 473 tissue engineering research. *Clin Orthop* 1999;367(Suppl.):S59-67.

474 [16] Elder SH, Kimura JH, Soslowsky LJ, Lavagnino M, Goldstein
 475 SA. Effect of compressive loading on chondrocyte differentiation
 476 in agrose cultures of chick lim-bud cells. *J Orthop Res*
 477 2000;18(1):78-86.

478 [17] Ferguson C, Alpern E, Miclau T, Helms JA. Does adult fracture
 479 repair recapitulate embryonic skeletal formation. *Mech Dev*
 480 1999;87(1-2):57-66.

[18] Gardner TN, Stoll T, Marks L, Mishra S, Tate MK. The influence
 of mechanical stimulus on the pattern of tissue differentiation in a
 long bone fracture-an FEM study. *J Biomech* 2000;33:415-25.

[19] Hall BK. In vitro studies on the mechanical evocation of
 adventitious cartilage into bone. *J Exp Zool* 1968;168:283-305.

[20] Hall BK. Immobilization and cartilage transformation into bone
 in the embryonic chick. *Anat Rev* 1972;173:391-403.

[21] Hamrick MW. A chondral modeling theory revisited. *J Theor Biol*
 1999;201(3):201-8.

[22] Heegaard JH, Beaupre GS, Carter DR. Mechanically modulated
 cartilage growth may regulate joint surface morphogenesis. *J*
Orthop Res 1999;17(4):509-17.

[23] Heegaard JH, Carter DR, Beaupre GS. A mathematical model for
 simulating mechanically modulated growth in developing diar-
 throdial joints. *Trans Orthop Res Soc* 1995;20:73.

[24] Hunziker EB. Articular cartilage repair: are the intrinsic biological
 constraints undermining this process insuperable. *Osteoarthritis*
Cartilage 1999;7(1):15-28.

[25] Lehman WB, Atar D, Feldman DS, Gordon JC, Grant AD.
 Congenital pseudoarthrosis of the tibia. *J Ped Orthop* 2000;9:103-
 7.

[26] Luder HU. Frequency and distribution of articular tissue features
 in adult human mandibular condyles: a semiquantitative light
 microscopic study. *Anat Rec* 1997;248(1):18-28.

[27] Martin RB. Toward a unifying theory of bone remodeling. *Bone*
 2000;26(1):1-6.

[28] Murray RC, Zhu CF, Goodship AE, Lakhani KH, Agrawal CM,
 Athanasiou KA. Exercise affects the mechanical properties and
 histological appearance of equine articular cartilage. *J Orthop Res*
 1999;17(5):725-31.

[29] Probst A, Spiegel HU. Cellular mechanisms of bone repair. *J*
Invest Surg 1997;10(3):77-86.

[30] Wu QQ, Chen Q. Mechanoregulation of chondrocyte prolifera-
 tion, maturation, and hypertrophy: ion-channel dependent trans-
 duction of matrix deformation signals. *Exp Cell Res*
 2000;256(2):383-91.

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