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The scope of this grant proposal is to investigate whether adhesion of prostate cancer cells to extracellular matrices regulates cell growth and survival. We have shown that attachment of LNCaP cells to a variety of matrices results in the phosphorylation of the focal adhesion kinase(FAK) and the scaffolding protein, p130. Downstream effectors of this signalling pathway in LNCaP cells include the Crk family of proteins, and the Crk binding proteins, C3G and DOCK180. Therefore upon adhesion of LNCaP cells, signalling pathways to the nucleus and cytoskeleton become activated to cause cell proliferation and cytoskeletal reorganization. We have set up a proliferation assay and measured a 15 - 20% increase in cell proliferation upon adhesion of LNCaP cells to fibronectin, collagen I and matrices generated by cultured osteoblastic cells. In addition, adhesion also prolonged the survival of LNCaP cells. Our results demonstrate, that adhesion to the substratum plays an important role in the regulation of proliferation and survival of LNCaP cells.

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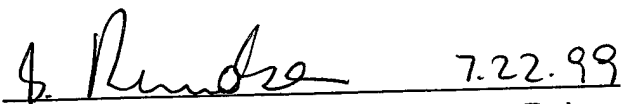

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TABLE OF CONTENTS

INTRODUCTION:

Adhesion of prostate cancer cells to extracellular matrix proteins regulates cell proliferation and cell survival (Giancotti, 1997). We therefore proposed to investigate the basic mechanisms that are responsible for tumor growth as a consequence of prostate cancer cell adhesion to different matrices. Integrins function as adhesion receptors at the cell – matrix interface through their binding to extracellular matrix proteins (Assoian and Zhu, 1997). Therefore signaling pathways that promote cell proliferation and survival originate from the cytoplasmic domain of integrins and associated molecules. Binding of cells to matrix proteins elicits the transmission of signals from the cell surface to the nucleus and regulates the cell cycle and the transcription of genes related to cell survival. The focal adhesion kinase (FAK) is intimately associated with activated integrins and through binding and phosphorylation of its substrates FAK initiate several signal transduction pathways (Guan, 1997). One of these pathways involves the scaffolding protein, p130Cas. The central role of p130Cas in oncogenesis has been well documented in *Src* and *Crk* transformed cells (Birge et al. 1992). Therefore the phosphorylation of p130Cas in response to cell adhesion and the activation of downstream signaling pathways constitutes the main goal of this grant proposal.

BODY:

- Research accomplishments for Task 1 "Involvement of Crk proteins and their ligands in integrin mediated signal transduction events"

a. Activation of FAK and phosphorylation of p130Cas upon cell adhesion: We have established a system to investigate adhesion dependent phosphorylation events in LNCaP cells. Cells are trypsinized and the trypsin is neutralized by addition of soy bean trypsin inhibitor in the absence of serum proteins. Trypsinization will remove integrin bound ligands and lead to dephosphorylation of some tyrosines withinp130Cas (fig. 1).

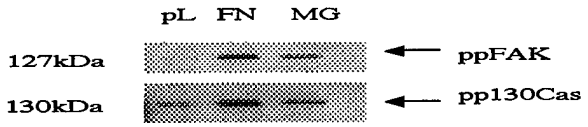

Figure 1 Phosphorylation of FAK or p130Cas upon adhesion of cells to polylysine (pL), fibronectin (FN) or matrigel (MG). Proteins were visualized with an anti-phosphotyrosine antibody.

Upon adhesion of LNCaP cells to matrix proteins, FAK becomes phosphorylated. P130Cas remained tyrosine phosphorylated, but LNCaP cell adhesion leads to specific de-novo phosphorylation of tyrosine residues that are binding sites for the CrkSH2 domain. In immunoprecipitation experiments, we showed specific precipitation of p130Cas by a gst-Crk SH2 construct. Two forms of p130Cas exist in LNCaP cells. The larger form has the expected size of 130kDa, but the second, smaller form corresponds to HEF1. Upon cell adhesion, only p130Cas binds the CrkSH2 domain. Surprisingly, in cultured adherent cells, both proteins bind the CrkSH2 domain. Thus, it is possible that HEF1 only becomes tyrosine phosphorylated upon prolonged adhesion and not in the four hour time course routinely used in our study.

b. **In-vivo complex formation of Crk and CrkL with upstream and downstream signaling proteins:** In co-precipitation experiments, we showed stable inducible complex formation between Crk/CrkL and p130Cas upon cell adhesion (fig. 2a). In contrast, the binding of Crk/CrkL to C3G also occurred in nonadherent cells (fig. 2b). It is therefore most likely that the phosphorylation of p130Cas is the regulatory step in this signaling pathway and leads to the translocation of Crk/CrkL complexes to sites of integrin signaling. Even though LNCaP cells express about equal amounts of Crk and CrklL, there was no reproducible difference between Crk and CrkL binding to upstream or downstream ligands in LNCaP cells. The binding of Crk/CrkL to Dock180 could not be demonstrated due to the poor quality of the Dock180 antibodies. However we could demonstrate the presence of DOCK180 in LNCaP cells.

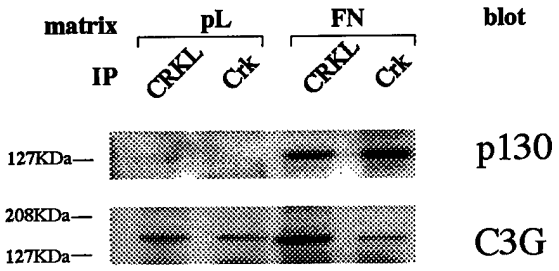

Figure 2. Co-precipitation of Crk or CrkL and p130Cas or C3G from cell lysates of cells adhered to polylysine (pL) or fibronectin (FN)

c. **Adhesion mediated cell proliferation assay for LNCaP cells:** We measured incorporation of BrdU to determine the number of cycling cells. The proliferative effects of different matrices and individual matrix proteins in the absence of serum derived growth factors were first analyzed. Conditions for this assay were established and resulted in a reproducible 10-15% baseline BrdU incorporation in LNCaP cells. In LNCaP cells grown on various matrices, BrdU incorporation was increased to 30 – 40% (fig. 3). These results clearly show that matrix proteins alone exert a mitogenic stimulus in LNCaP cells.

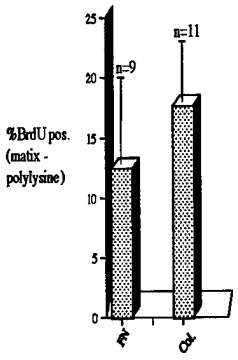

Figure 3. Proliferation assay of LNCaP cells on fibronectin (FN) and collagen (col) coated surfaces. The percent of BrdU positive cells was calculated. The proliferation on polylysine was subtracted as the background, so that the bars reflect the increase in proliferation of LNCaP cells on FN or col.

d. **Anoikis assay:** Cell adhesion likely plays a critical role in the survival of LNCaP cells. Accordingly, we documented the onset of DNA fragmentation in suspended cells after 24 – 48 hrs. We therefore wanted to measure jun-kinase activity in these suspended cells. However, at the time of these experiments, jun-kinase activation was difficult to measure, since we relied on the endogenous levels of jun-kinase in LNCaP cells. Recently, phospho-antibodies have become available that can be used to assess the activation of endogenous jun-kinase. We will repeat the anoikis experiments using these new antibodies to determine the role of jun-kinase in anoikis of LNCaP cells.
e. **Activation of Rap1A:** Rap1A becomes activated upon growth factor stimulation of LNCaP cells as recently demonstrated (Posern G et al., 1998)). LNCaP cells express Rap1A and B-Raf whose activation can lead to MAPK activation. Therefore it is possible that in LNCaP cells, Rap1A does not mediate cell cycle arrest, but rather cell proliferation. In addition, Rap1A was shown to become activated upon cell adhesion of fibroblasts (ref.). In order to determine the activation of Rap1A in LNCaP cells upon cell adhesion, we obtained a gst-RGD construct. This construct consists of the Rap binding domain of RalGDS. This gst-fusion protein specifically binds activated Rap1A, but does not bind Rap:GDP or Ras. We are in the process to set up the precipitation assay that is needed to investigate activation of Rap1A in LNCaP cells.
f. **Activation of the MAPK pathway:** Tested the phosphorylation of SHC and MAPK in LNCaP cells upon stimulation by EGF. Both proteins became phosphorylated within 10min of EGF treatment. Since SHC migrates close to the IgG heavy chain on SDS gels, we used anti-phosphotyrosine antibodies coupled to agarose to precipitate phosphorylated SHC complexes. This technique will need to be refined to study SHC activation upon cell adhesion.

In summery we have accomplished our goals listed under Task 1 of the grant proposal.

The main criticism of the reviewers regarding this grant proposal was the usage of prostate cancer cell lines that may not reflect the real situation of primary prostate cancer cells. Because of this deficiency, the relevance of the proposed experiments to prostate cancer research was questioned. Therefore we decided to extend our studies within the scope of this grant to address questions that are related to the biologic behavior of prostate cancer. Specifically, we have focused on the effects of the extracellular matrix synthesized by cultured bone marrow stromal cells. This extracellular matrix elicits mitogenic as well as a survival related signals in adherent LNCaP cells. We therefore decided to study the proposed signaling pathways during the adhesion of LNCaP cells to stromal cells matrix. The original statement of work remains the same but the system became applicable to the growth regulation of metastatic prostate cancer in the bone marrow environment.

RESEARCH ACCOMPLISHMENTS:

- Adhesion assay for LNCaP cells on different matrices from bone marrow stromal cells, osteoblast cell lines and commercial matrix constituents
- Demonstration of FAK and p130Cas phosphorylation and complex formation with dowstream effector proteins by co-immuneprecipitation
- Proliferation assay for matrix adherent LNCaP cells through incorporation of BrdU.
- Demonstration of mitogenic properties of individual extracellular matrix proteins and intact matrices on LNCaP cells
- Anoikis assay and Jun-kinase activation assay
- Assays for activation of Rap1A in LNCaP cells

REPORTABLE OUTCOMES:

- A manuscript entitled "Adhesion of LNCaP cells in the bone marrow environment" is under preparation
- The proposed work will provide the basis for submission of an RO1 grant in October 1999.

CONCLUSIONS:

Adhesion of LNCaP cells to a variety of matrices results in the phosphorylation of the focal adhesion kinase (FAK) and the scaffolding protein, p130Cas. Downstream effectors include the Crk family of proteins, C3G and DOCK180. Therefore upon adhesion of LNCaP cells, signaling pathways to the nucleus and the cytoskeleton become activated to cause cell proliferation and cytoskeletal reorganization. Attachment of LNCaP cells to extracellular matrix proteins also stimulates cell proliferation. It is therefore likely, that the substratum plays an important role in controlling the proliferation and survival of prostate cancer cells.

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