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13. ABSTRACT (Maximum 200 Words)

Osteonectin is a secreted matrix protein that has a variety of functions, including cell adhesion modulation and chemoattraction. It has been implicated as a chemoattractant for breast cancer cells despite the evidence that some cancer cells secrete osteonectin. Other studies demonstrate the ability of osteonectin to permeabilize a vascular endothelial cell layer even though some endothelial cells also secrete osteonectin. We hypothesize that there are differences between cancer-derived osteonectin, bone-derived osteonectin, and vascular endothelial-derived osteonectin. Osteonectin was separated by SDS-PAGE under reducing and non-reducing conditions followed by immunoblotting; a small variance in molecular size between osteonectin from different sources is detected. Results suggest that breast cancer cells secrete two types of osteonectin with different configurations of disulfide linkages, whereas bone cells secrete osteonectin with a single pattern of disulfide links. No differences were observed from deglycosylation and phosphorylation assays while osteonectin-induced endothelial permeabiliztion was also not detected by interference reflection microscopy. The research completed in the last year has supported the hypothesis that there are structural differences between breast cancer-secreted and vascular/bone-secreted osteonectin.

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Introduction

Osteonectin is a secreted matrix protein that has a variety of functions, including cell adhesion modulation and chemoattraction. It has been implicated as a chemoattractant for breast cancer cells despite the evidence that some cancer cells secrete osteonectin. Other studies demonstrate the ability of osteonectin to permeabilize the vascular endothelial cell layer even though some endothelial cells also secrete osteonectin. This research aims to examine the differences between cancer-derived osteonectin, bone-derived osteonectin, and vascular endothelial-derived osteonectin. Of key interest is that these differences may contribute to the affinity of breast cancer cells to bone. During the past year of research, structural and functional differences between the two types of osteonectin have been analyzed.

Body

Task 1 involves analysis of structural differences between osteonectin of different sources. We separated osteonectin by SDS-PAGE under reducing conditions followed by immunoblotting; a small variance in molecular size between osteonectin from different sources is detected. Two bands from the MDA-MB-435 (~41 and 38 kDa) and MDA-MB-468 (~41 and 36 kDA) cell lines under non-reducing conditions (previously reported) appear as a single band (~57 and 54 kDa, respectively) when reduced; this phenomenon is not observed in the bone (hFOB1.19) or endothelial-derived (HBME-1) osteonectin which display a only a single band under both reducing (~53 kDa) and non-reducing (~38kDa) conditions (Figure 1). These results suggest that breast cancer cells secrete two types of osteonectin with different configurations of disulfide linkages, whereas bone cells secrete osteonectin with a single pattern of disulfide links. Because disulfide bonds influence protein folding, at least one of the breast cancer-secreted types of osteonectin will have a different three-dimensional structure from the bone or endothelial-derived osteonectin.

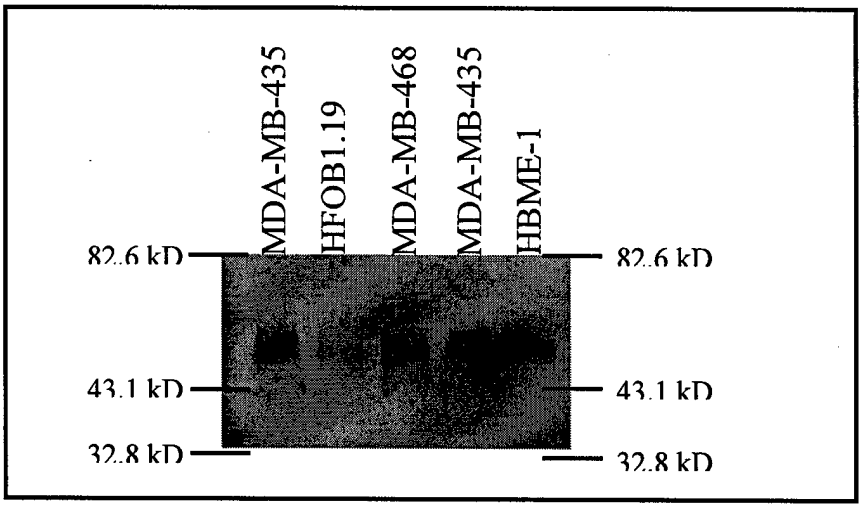

Figure 1. Comparison of molecular sizes of osteonectin from breast cancer cells and bone cells by immunoblotting. Conditioned serum–replacement medium (Sigma) was collected after 48 hours and separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose and then immunoblotted. Osteonectin from MDA-MB-435

cells is ~57 kDa while MDA-MB-468 is ~54 kDa. Osteonectin from HBME-1 and hFOB cell lines was ~53 kDa..

Task 1A and 1B have now been completed and were reported mainly last year. Task 1C, investigation of glycosylation differences between breast cancer-derived and bone-derived osteonectin, was further analyzed by use of an enzymatic deglycosylation assay. Contrary to previous results using a different assay system which included the presence of tunicamycin, all sources of osteonectin have similar levels of glycosylation. The osteonectin from HBME-1, hFOB, MDA-MB-435, and MDA-MB-468 cell lines lack sialic acid and seronine/threonine linked glycosyl groups. All tested cell lines produced osteonectin with N-linked glycosylation (Figure 2). These results contradict last year's finding that the breast cancer cell line-derived osteonectin lack N-linked glycosylation as determined by tunicamycin assays. Further examinations led to the finding that the tested breast cancer cell lines are resistant to tunicamycin resulting in inaccurate data.

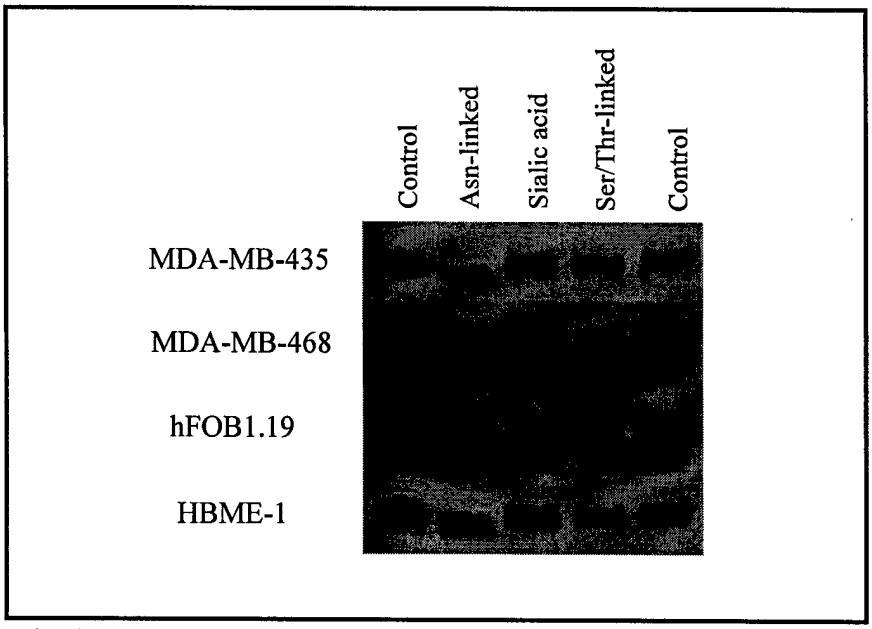

Figure 2. Comparison of molecular size of deglycosylated osteonectin. Osteonectin from all cell types have N-linked glycosylation while sialic acid and Ser/Thr-linked glycosylation are absent. Affinity purified osteonectin was treated with an enzymatic deglycosylation kit (Sigma), separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted.

Literature suggests that osteonectin has multiple serine sites for possible phosphorylation (Fisher et al., 1987); therefore analysis of phosphoserines has been conducted to support Task 1. Osteonectin was purified by an affinity column, transferred to nitrocellulose and immunoblotted for phosphorylated serine. Preliminary data suggests that none of the collected osteonectin from HBME-1, hFOB, or the breast cancer cell lines contain phosphorylated serine groups.

Task 1D, test osteonectin for affinity to other matrix proteins, is presently being conducted. The initial tests have included osteonectin binding to collagen type I, collagen type IV and fibronectin. Future assays will be done on Nunc Maxisorb 96-well plates; our laboratory has purchased a plate washer that will result in more accurate and consistent data.

In preliminary research for Task 2, we have been unable to replicate assays showing permeablilization of an endothelial layer due to the presence of osteonectin. Interference reflection microscopy on an HBME-1 cell layer treated with 1μg/ml of MDA-MB-435-derived osteonectin failed to show any changes in cell shape, permeablilization or loss of focal adhesion complexes (Figure 3). The study indicates that breast cancer-derived osteonectin does not influence vascular endothelial cell adhesion. Immunocytochemistry for actin redistribution and loss of focal adhesion complexes (through loss of vinculin or talin) need to be conducted.

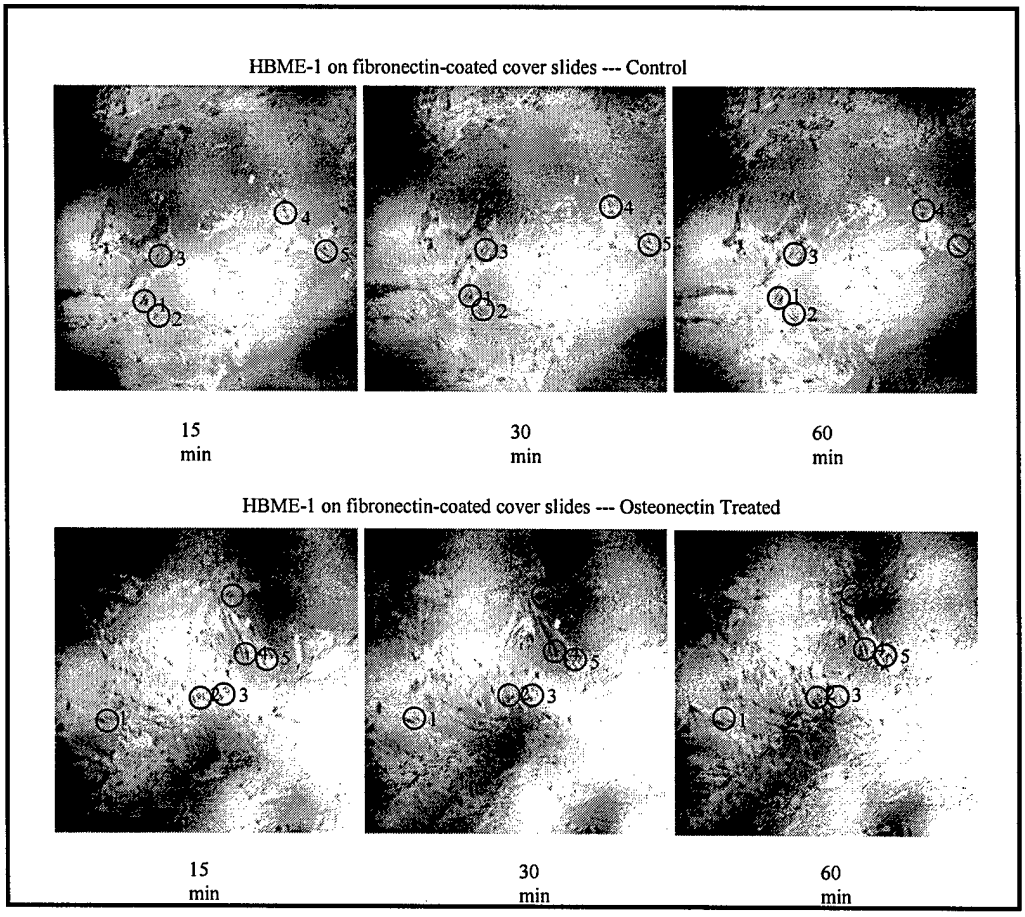

Figure 3 . Comparison of focal adhesion complexes in the presence and absence of osteonectin. After an hour with +/- 1μg/ml MDA-MB-435 osteonectin treatment, the number of focal adhesions in HBME-1 cells did not change. Glass cover slides are coated with 20μg/ml fibronectin (Biomedical Technologies Inc., Stoughton, MA).

HBME-1 cells were grown to confluence, treated with either 0 or 1μg/ml MDA-MB-435 osteonectin.

Key Research Accomplishments

1. Determined differences in disulfide linkage patterns between osteonectin from breast cancer cells compared to osteonectin of bone or vascular endothelial cells.
2. Osteonectin from breast cancer, osteoblasts and vascular endothelial cells do not have sialic acid or Ser/Thr linked glycolyl groups.
3. Osteonectin from breast cancer, osteoblasts and vascular endothelial cells have N-linked glycosyl groups.
4. Preliminary data suggests that osteonectin from HBME-1, hFOB or two breast cancer cell lines do not contain phosphorylated serine sites.
5. Loss of focal adhesion plaques or induced endothelial permeabiliztion due to the presence of bone-derived osteonectin was not detected by interference reflection microscopy.

Reportable Outcomes

Activities supported by this award this year included finishing all of my course requirements for completion of my degree. Also, in August of 2003 I passed the comprehensive exam. I participated in a weekly journal club in which current literature on breast cancer, bone-related knock-out discoveries, and cell communication was presented and discussed by faculty and other graduate students. Each semester I attend, typically on a weekly basis, on-campus seminars dealing with a variety of disciplines such as neurobiology, cancer, developmental biology, cell biology, nutrition, and careers in science. I participated in the "American Association of Cancer Research" meeting in Washington D.C, the "Fourth North America Symposium on Skeletal Complications of Malignancy" in San Antonio Texas, and the "Women in the Sciences and Engineering VOICES" conference in State College Pennsylvania; this gave me the opportunity to meet and interact with other scientists in the fields of cancer and bone. In the laboratory, I have mastered many new techniques including ELISA development for the substrate binding assays, protein analysis by immunoblotting and enzymatic treatments, confocal microscopy, and interference reflection microscopy.

Conclusion

The research completed in the second year supports the hypothesis that there are structural differences between breast cancer-secreted and bone-secreted osteonectin. This has been demonstrated by size analysis on western blots and the conclusion that different arrangements of disulfide linkages exist. Because disulfide bonds influence protein folding, at least one of the breast cancer-secreted types of osteonectin will have a different three-dimensional structure from the bone or endothelial-derived osteonectin. The past year of research will further identify structural variation and better understand the effects osteonectin has on living cells.

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Fisher LW, Hawkins GR, Tuross N, Termine JD. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem. 262 (20): p 9702-9708, 1987.