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The present research project consists of two major parts. In the first part, we examined the hypothesis that CD44 allows tumor cells to bind and degrade hyaluronan and this, in turn, stimulates their vascular supply. In Tasks 1 through 4, we investigated this hypothesis by examining the effects of CD44 expression on tumor progression. We found that both primary and secondary tumors are heterogeneous with respect to the expression of CD44 and hyaluronan, both of which are associated with dividing cells on the surface. However, we could not find any obvious correlation between the distribution of HA and blood vessels (endothelial cells). Clearly, more research is needed to test the working hypothesis.

In the second part, we have explored the possibility of targeting lung metastases with proteins from cartilage (PG) that can bind to hyaluronan that is closely associated with these tumor cells. We have examined the feasibility of this approach in Tasks 5 through 7 by testing the ability of the PG complex to gain access to lung metastases and to deliver chemotherapeutic drugs to cultured tumor cells. Thus far, the results have been encouraging.

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Charles Underhill 9/28/97

PI - Signature Date

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INTRODUCTION:

Nature of the Problem:

The present research project is concerned with the interaction between tumor cells and HA (HA), one of the major components of the extracellular matrix. HA is a very large, negatively-charged carbohydrate that functions to maintain the extracellular space. In previous studies, we have shown that the degradation of HA is mediated by a cell surface glycoprotein termed CD44 (also known as the HA receptor). This protein functions to bind HA to the cell surface so that it can be internalized and then degraded by lysosomal enzymes. We have found that this degratory process can be prevented by antibodies which block the interaction between CD44 and HA.

The working hypothesis of the present application is that this CD44-mediated degradation of HA enhances tumor progression by increasing their vascular supply. This hypothesis is supported by the following lines of evidence. First, a number of studies have shown that the expression of CD44 is causally associated with the metastatic process. For example, transfection of cells with CD44 expression vectors stimulates their metastatic properties. Secondly, human breast cancer cell lines that express CD44 can degrade HA. Thirdly, the fragments of HA produced in the process of degradation have angiogenic properties leading to increased vascularization. And fourthly, large amounts of HA surround many types of blood vessels, and the degradation of this HA by tumor cells would increase their vascular supply.

Background of Previous Work:

General Characteristics of CD44: CD44 defines a family of cell surface glycoproteins which has been implicated in cellular processes such as adhesion, migration, lymphocyte homing and tumor metastasis (1, 2). These proteins are found on a variety of cell types including epithelia, leukocytes, and tumor cells. As a result of alternative splicing and variations in the degree of glycosylation, members of the CD44 family come in several different molecular weight forms, ranging from 80 to well over 200 kDa (2).

As illustrated in *Fig. 1,* CD44 may be divided into three domains, base upon both structural and functional considerations. First, the C-terminal domain of the molecule consists of the transmembrane and cytoplasmic region of the molecule. This region of the molecule can be associated with actin filaments, possibly through an ankyrin-like molecule, and this interaction may be modified by either phosphorylation or acrylation (6-8). The association with the cytoskeleton may be an important factor in determining the distribution of CD44 on the cell surface which, in turn, may influence it ability to interact with HA. Secondly, the middle domain of the molecule is highly glycosylated and in some cases may serve as an attachment site for either chondroitin or heparan sulfate side chains, which are responsible for the

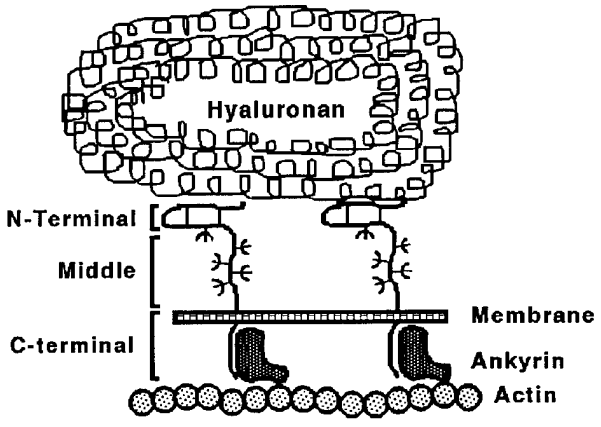

Fig. 1. Model of CD44 and its interactions with the cytoskeleton and HA.

interactions with collagen and fibronectin (9-11). This region of the molecule shows considerable variation in sequence due to alternative splicing of the mRNA. Already, at least 15 isoforms of CD44 have been identified, and most of the different inserts occur in this middle domain (12). And thirdly, the N-terminal domain shares sequence homology with link protein of cartilage and is responsible for the binding of HA. This region recognizes a six sugar sequence of HA, but will also bind chondroitin sulfate with a lower affinity (1, 2).

Involvement of CD44 in Tumor Progression: Recently, several lines of evidence have suggested that CD44 is involved in tumor metastasis. For example, a number of studies have found that high levels of CD44 are associated with certain types of carcinomas, high grade gliomas and many non-Hodgkin's lymphomas (17-19). In the case of lymphomas and other tumors, large amounts of this protein are correlated with the rapid dissemination and negative prognosis of these tumors (18, 19). In preliminary studies, we have also found that the expression of CD44 by a panel of human breast cancer cell lines is correlated with their metastatic behavior as measured by a variety of *in vitro* assays.

More direct evidence that the expression of CD44 is related to the metastatic behavior of tumor cells comes from the work of Gunthert and his associates (20). They found that highly metastatic rat pancreas cell lines express a particular isoform of CD44 (termed CD44v), which was absent from their non-turmorigenic counterparts. More importantly, when non-metastatic cells were transfected with cDNA for this CD44 isoform, they were converted into a more metastatic phenotype (20). In addition, antibodies directed against this particular isoform of CD44 blocked tumor metastasis in experimental models (20). These observations suggest that CD44v is responsible for the metastatic behavior of these cells.

Other isoforms of CD44 also appear to influence the metastatic behavior of cells. Sy et al. (21) have shown that when human lymphoma cells were transfected with the cDNA for a 85 kDa isoform of CD44 which binds HA, there was a marked increase in tumor formation and metastatic behavior, while transfection with an isoform that cannot bind HA had no such effect. In addition, the growth of these tumors *in vivo* could be blocked by co-injection of a soluble form of CD44, which presumably acted by competitively inhibiting the interactions of CD44 with its ligand, HA (22). These researchers also noted that lymphoma cells lacking CD44 also formed both primary and metastatic tumors, albeit at a lower rate. Based on these results, these researchers concluded that expression of the 85 kDa form of CD44 promotes, but is not required for, tumor growth and metastasis (21).

However, none of the studies described above address the mechanism by which CD44 promotes tumor progression. This question is one of the major goal of the present research project.

Role of CD44 in Degradation of HA: One possible mechanism by which CD44 could influence the behavior of tumor cells is by mediating the degradation of HA. Indeed, in earlier studies, we have shown that CD44 is critically involved in the uptake and degradation of HA by both transformed fibroblasts (SV-3T3 cells) and alveolar macrophages (23). To demonstrate this phenomenon, we cultured these cells in the presence of [^{3}H] HA. After various lengths of time, the cultures were digested with pronase to release the HA, and the fragments of [^{3}H] HA were separated from the macromolecular HA by centrifugation through size specific membranes (Cetricon 30 Micro concentrators). Both the SV-3T3 cells and the macrophages degraded significant amounts of the HA. Examination of the digests by molecular-sieve chromatography revealed that the resulting fragments ranged in size from monosaccharides to higher oligosaccharides; smaller fragments were not detected.

CD44 was clearly involved in the degradation of HA, since this process was almost completely blocked by the K-3 mAb against CD44. Furthermore, the degradation was also blocked by the addition of an excess of non-labeled HA, while the addition of other glycosaminoglycans such as dermatan sulfate, chondroitin-4-sulfate and heparin had only a small inhibitory effect (23). This was in keeping with previous studies indicating that CD44 binds with relative specificity to HA as compared to other glycosaminoglycans (1). Similarly, oligosaccharide fragments of HA smaller than a hexasaccharide had only a modest inhibitory effect on the degradation, which is consistent with the size specificity for recognition by CD44 (1).

Collectively, the above results indicated that CD44 plays a key role in the degradation of HA. More specifically, CD44 is responsible for the initial binding of HA to the cell surface so that it can be internalized and degraded by acid hydrolases (see model in *Fig. 2*). This CD44-mediated uptake is consistent with previous studies suggesting that CD44 is associated with the cytoskeleton (6). Thus, the degradation of HA takes place in a fashion similar to that of other receptor-mediated degradatory processes such as LDL and transferrin.

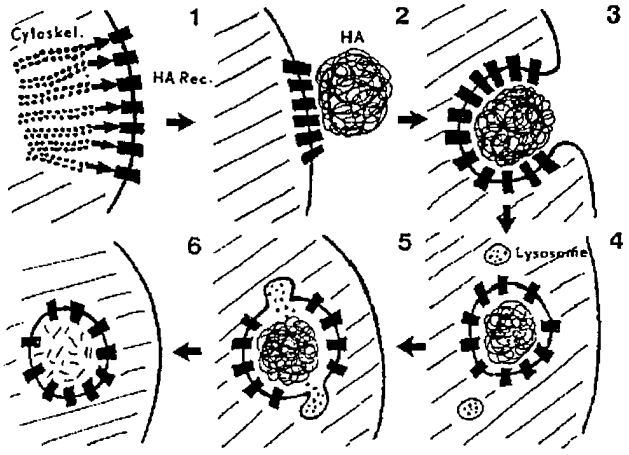

Fig. 2. Model of HA degradation. (1) Initially molecules of CD44 are clustered on the cell surface through their interaction with the cytoskeleton. (2) A number of molecules of CD44 bind simultaneously to a molecule of HA. (3 and 4) The HA is endocytosed into a vesicle. (5) Lysosomes fuse with the vesicle. (6) The HA is degraded by the action of acid hyrolases.

The ability of cells expressing CD44 to degrade HA may be important during normal processes of tissue morphogenesis and cell migration. For example, during the development of the lungs, there is a progressive decrease in the amount of HA in relation to protein content (24). The decrease reflects the loss of interstitial tissue so that gas exchange can take place at the time of birth. We found that this loss of HA was inversely correlated with the number of macrophages expressing CD44, which increased in number during embryonic development. In addition, histochemical staining revealed that some of these macrophages contained HA in their cytoplasm, suggesting that macrophages had internalized HA from the extracellular matrix. This possibility was further supported by the fact that when new-born mice were injected with the KM-201 monoclonal antibody, which blocks the interaction between HA and mouse CD44, the number of HA-containing macrophages in the lungs decreased while the concentration of HA increased. Taken together, these results suggest that macrophages can internalize HA during lung development and could possibly play a significant role in its removal (24).

CD44 and HA of Human Breast Cancer Cell Lines: In preliminary studies, we have examined the relationship between CD44 expression and the binding and degradation of HA in a panel of human breast cancer cell lines (26). These cell lines have been previously characterized for various markers of invasive potential and represent a convenient *in vitro* model system for studies of breast cancer progression (27).

In general, the cell lines that expressed the most CD44 were also the most invasive, as judged by *in vitro* assays. For example, the Hs578T cell line that expressed the greatest amount of CD44 was invasive, as

judged by migration and chemotaxis in Boyden chamber assays, while the ZR-75-1 cell line, that did not express detectable levels of CD44, was judged to be non-invasive in both of these assays. Similarly, the expression of high amounts of CD44 was generally associated with the lack of estrogen receptors and the presence of the intermediate filament protein vimentin, both of which have been shown to indicate a poor prognosis in human breast cancer (27). This trend is consistent with other studies indicating that the expression of CD44 is correlated with metastatic behavior of tumor cells (17-19)

We then examined the ability of these cells to degrade HA. For this, the cells were cultured in the presence of [^{3}H]HA, and after 40 hours, the resulting fragments were detected using Centricon 30 micro concentrators. The degradation of HA was closely correlated with the amount of CD44 (correlation coefficient, $r = 0.951$). In general, the cell lines that expressed the most CD44 also could degrade the most HA. This correlation was remarkably good, considering the fact that other factors are clearly involved in the degradation process, such as the rate of endocytosis and the amount of lysosomal HAase. The involvement of CD44 in HA degradation was further supported by the observation that Hermes-1 mAb, which is directed against an epitope close to the HA binding domain of CD44 (3), blocked the degradation of HA.

We then examined the distribution of HA in xenografts formed by these cell lines in nude mice. For this, the cell lines were injected into the fat pads of nude mice, and the resulting xenografts were histochemically stained for HA, using a specific probe derived from cartilage. One feature common to all of the grafts was that HA was a prominent component of the matrix at the junction between the graft and the surrounding normal tissue. In some cases, the demarcation boundary between graft and the surrounding tissue was diffuse, while in others it was relatively sharp. The type of boundary differed both from tumor to tumor and within a single tumor.

Significant differences were observed in the distribution of HA within the body of the tumor xenografts. In the grafts of cells that expressed low levels of CD44, HA was generally a major component of the interstitial matrix. In contrast, in the body of grafts formed by cells that expressed high levels of CD44, HA was greatly reduced or absent. These grafts were relatively deficient in interstitial connective tissue and had a more homogenous appearance. The one exception to this correlation was the MDA-468 cell line, in which the amount of HA varied significantly from region to region. However, in general, the expression of CD44 was inversely correlated with presence of HA within the body of the tumor cell xenografts. We speculate that this difference is due to the ability of CD44 expressing tumors to degrade the HA.

Effect of HA Degradation on Vascularization: The central question being addressed in this research project is how does CD44 enhance the metastatic activity of tumor cells. Based upon a variety of evidence, we speculate that the CD44-mediated degradation of HA lead to an increase in the blood supply to the tumor cells which enhances their growth rate as well as their ability to survive and form metastases. This postulated increase in blood supply may occur through two different mechanisms, which may occur simultaneously.

First, the oligosaccharide fragments of HA produced as a by-product of HA degradation may stimulate the formation of new blood vessels. Indeed, studies have shown that oligosaccharide fragments of HA have angiogenic properties. For example, West and coworkers found that fragments of HA 3 to 16 disaccharides in length stimulate the formation of blood vessels when applied to the chick chorioallantoic membrane (28). In contrast, macromolecular HA and fragments of other glycosaminoglycans (chondroitin-

4 and 6-sulfate) were ineffective, suggesting that the effect is specific for HA. These workers went on to show that these oligosaccharide fragments of HA also stimulated the proliferation of endothelial cells in tissue culture (29). This effect appeared to be restricted to endothelial cells since fibroblasts and smooth muscle cells were not effected by these fragments. Presumably, the endothelial cells contain a receptor that can detect fragments of HA. This receptor is probably distinct from CD44 which in most cases is not present on endothelial cells. Along these lines, Banerjee and Toole (30) have shown that antibodies against an HA binding protein on the surface of endothelial cells blocks the migration of these cells. Thus, it is possible that tumor cells expressing CD44 could release fragments of HA which interacts with receptors on the surfaces of endothelial cells and stimulate the formation of new blood vessels.

A second possible mechanism is that tumor cells expressing CD44 can degrade the HA surrounding blood vessels. In histochemical studies, we have examined the distribution of HA surrounding blood vessels in different tissues. In some tissues, such as the liver and spleen, only small amounts of HA are associated with the blood vessels. In contrast, in other tissues such as the dermis, the lamina propria of the intestinal track, the stroma of the lungs and the pericardium of the heart, large amounts of HA are associated with the blood vessels. In these tissues, HA was generally associated with the intima of veins and venules, immediately beneath the endothelial cell lining. In contrast, in arteries, it was generally reduced or absent from the intima, but was present in the adventitia. Thus, the ability of tumor cells to degrade this HA could allow them to get in closer proximity to the blood supply and consequently receive more nutrients. Along these lines, it is also possible that these tumor cells could more easily penetrate the blood vessels, enter the circulation and metastasize to different locations.

We further hypothesize that regardless of the mechanism, the increase in the blood supply results in a selective advantage for those cells that express CD44. When we stain normal mouse mammary tissue for CD44, we find that only small amounts of it are expressed on the ductal cells. However, in primary tumors of transgenic mice, we find that the expression of CD44 is variable. It is present in some regions but absent from others. We speculate that the CD44 expressing cells of the primary tumor are at a selective advantage for giving rise to metastases. One of the specific aims of this research project is to determine if the metastases that arise from these mixed primary tumors have a high probability of expressing CD44.

Purpose of the Present Work:

There are two major aspects of this present progress report. In the first part we will test the working hypothesis that the expression of CD44 allows tumor cells to degrade HA, which, in turn, results in an increase in the blood supply. This increase may occur by formation of new blood vessels induced by fragments of HA, and/or by the degradation of HA present around preexisting blood vessels, which improves the access of the tumor cells to the blood supply. In either case, the increase in the blood supply imparts a selective advantage to the tumor cells that express CD44. As a result, while primary tumors may be heterogeneous with respect to the expression of CD44, secondary tumors will have much higher probability of expressing this molecule. This working hypothesis will be examined in Tasks 1 through 4.

The second part of this report is concerned with the implications of an observation that we made during the previous year. We found that tumors that have metastasized to the lungs are associated with large amounts of HA in the surrounding tissue. This HA may have been produced by the normal lung tissue as part of an immune response. Regardless of its origins, this HA could serve as a marker for tumor cells present in the lung tissue. Furthermore, it may be possible to specifically target this HA using a complex of proteins isolated from cartilage termed PG. The PG complex may have a number of advantages over other

probes to target tumors such as antibodies. First, it is not highly antigenic since the HA binding proteins are highly conserved between species and are linked with large amounts of glycosaminoglycans which insulate the core protein. Secondly, large amount of the complex can be prepared. And finally, the large size will also allow large amount of chemotherapeutic agents to be attach to it that increases its potential effectiveness as a drug delivery system. The feasibility of this approach will be examined in Tasks 5 through 7.

Methods of Approach:

1. Examine the effect of CD44 expression of the vascularization of tumors: To determine if the expression of CD44 leads to an increase in the vascular supply, we will transfect a human breast cancer cell line with a CD44 expression vector, and inject these cells into nude mice and allow them to grow. The resulting xenografts will be and examined for the presence of blood vessels. If our hypothesis is correct, then xenografts derived from CD44 positive cells should be associated with a greater number of blood vessels than the CD44 negative cells.

2. Determine the effects of various agents on the vascularization of tumors expressing CD44: Osmotic pumps that release either control or blocking antibodies to CD44 will be implanted subcutaneously in nude mice along with tumor cell lines that express CD44. After a period of growth, the xenografts will be removed and examined histologically for blood vessels. If our working hypothesis is correct, then the blocking antibodies should inhibit the vascularization of the tumor cells.

3. Examine the expression of CD44 in primary and secondary tumors of transgenic mice: According to our working hypothesis, the expression of CD44 imparts a selective advantage to cells with regard to tumor progression. To test this possibility, we will examine both primary and secondary tumors formed by transgenic strains of mice that spontaneously develop breast tumors. The xenografts will be analyzed histochemically for endothelial cells.

4. Survey specimens of human breast tumor for the presence of CD44, HA and vascular endothelial cells: To determine the significance of CD44, HA and endothelial cells in evaluating its metastatic potential, we will examine specimens of human breast cancer. If this pilot experiment shows a good correlation between these parameters, then we will expand this study to include a greater number of samples.

5. To examine various types of lung metastases for the expression of HA: In previous experiments, we observed that HA was often associated with lung metastases. To determine if this phenomenon is universal, we will examine a number of other systems involving metastases to the lungs.

6. To test the possibility of using PG to target lung metastases: To determine if the tumor-associated HA can be targeted with PG, we will inject a biotinylated form of this complex (b-PG) into mice that had tumor metastases. We then examined the lungs of this mouse to determine if the b-PG had gained access to the HA associated with the lung metastases.

7. To examine the effects of PG coupled to MTX on cultured tumor cells: To test the feasibility of using derivatives of PG to deliver chemotherapeutic drugs to tumor cells, we will couple methotrexate (MTX) to PG and test its ability to kill tumor cells in culture.

BODY

Introduction: During the previous year, the direction of the research has changed significantly. This was partially due to technical difficulties that have blocked progress on Tasks 1 and 2 (see below). More importantly, in Task 3, we make an interesting observation concerning the association of HA with tumor metastasis that forms the foundation of the new Tasks 5, 6 and 7. We found that when some types of breast cancer cells metastasize to the lungs, the surrounding lung tissue produces large amounts of HA. This phenomenon suggested the possibility of using this HA to target the tumors in the lungs with an HA-binding complex derived from cartilage that is composed of proteoglycan and link protein (PG). If successful, this binding complex could be used for determining the location of secondary tumor cells in the body and for delivering chemotherapeutic drugs to them.

To test the feasibility of using the HA-binding PG to target secondary tumors we have undertaken the following new studies: In Task 5, we examined a number of other lungs metastases to determine if the increase in tumor-associated HA was a universal phenomenon. In Task 6, we tested the possibility of using the PG complex to detect lung metastasis by injecting a biotinylated version of this complex into mice with xenografts of breast cancer. And finally, in Task 7, we coupled the chemotherapeutic agent methotrexate (MTX) to PG and tested its cytotoxic activity on cultured tumor cells.

Both the old and the new Tasks are discussed below.

Task 1: Examine the effect of CD44 expression of the vascularization of tumors: The purpose of this study was to test the hypothesis that CD44 enhances the vascularization of tumors. To accomplish this, we proposed to transfect human breast cancer cell lines with a CD44 expression vector, grow these cells in nude mice, and then analyze the resulting xenografts for endothelial cells.

Previous results: As described in the previous progress report, we transfected a number of human breast cancer cell lines (ZR-751, MCF-7, and ML-20) with an expression vector for human CD44. We characterized several clones of the transfected cells and found that they gained the ability to bind and degrade HA in a CD44-dependent fashion as we had predicted.

Results and Discussion: During this past year, we have injected these transfected cells into the fat pads of nude mice. Unfortunately, none of these transfected cells would grow in the nude mice despite repeated attempts under a variety of conditions (i.e. with and without Metragel and estrogen). Thus, we have been unable to test the hypothesis that the expression of CD44 allows the tumor cells to degrade the HA in the extracellular matrix and stimulate angiogenesis. We conclude from this that transfection with CD44 does not confer increased growth potential in nude mice, at least in the case of the human breast cancer cell lines. This is in keeping with the results from other laboratories that increased metastatic potential following transfection with CD44 expression vectors is very dependent upon the cell type being transfected. Thus, at this point, we have attempted this experiment with all of the appropriate human breast cancer cell lines at our disposal. While we have not given up on this approach, we are somewhat frustrated.

Recommendations: The only possibility left to us is to attempt this experiment with other cell lines. According, we propose to examine cell lines that are derived from different types of tumors (lung, prostate, colon etc.) and of a mouse origin instead of human. These cell lines must satisfy the following criteria: 1) they must not grow too aggressively in nude mice; and 2) they should express low levels of endogenous CD44.

Task 2. Determine the effects of various agents on the vascularization of tumors expressing CD44: The purpose of this set of experiments was to determine if vascularization of xenografts could be blocked by antibodies to CD44 or enhanced by fragments of HA or HAase. As described above, we have been unable to get the transfected cell lines to grow in the nude mice. Thus, no further progress (other than the preparation of reagents) has been made on this task.

Recommendations: Again, we must wait for progress in Task 1.

Task 3. Examine the expression of CD44 in primary and secondary tumors of transgenic mice: The purpose of this set of experiments was to compare primary versus secondary tumors with respect to the expression of CD44 and HA. For this, we examined a strain of mice that has been transfected with a polyomavirus middle T oncogene under the control of a mouse mammary tumor virus promoter/enhancer (31). This transgenic strain of mice forms multifocal mammary adenocarcinomas that metastasize to the lungs at a high frequency. The results of this study form the foundation of the new Tasks 5, 6 and 7.

Previous results: A mouse with a large tumor load was sacrificed and both the primary tumor and the lungs were removed and fixed overnight in formaldehyde. The tissues were then embedded in polyester wax, which helps to preserve the antigenicity (32) and then stained for both CD44 using the KM-201 mAb and HA using the b-PG probe. In both cases, the sections were incubated for one hour in the primary agent that was diluted in 10% calf serum, 90% saline. The sections were then incubated with peroxidase labeled streptavidin and finally a peroxidase substrate consisting of H_2O_2 and 3-amino-9-ethyl carbazole that gives rise to an intense red reaction product (33). The sections were then counterstained with Meyer's hemotoxylin that gives a blue color. The chromogens were preserved with Crystal/mount and then cover-sliped.

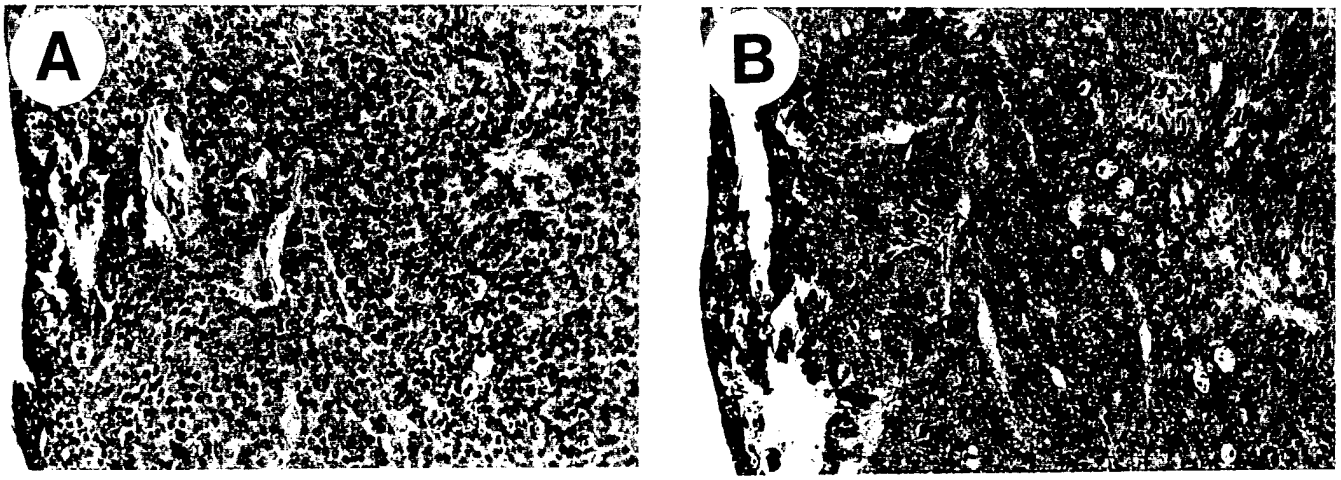

Fig. 3. Distribution of CD44 and HA in primary breast tumors from transgenic mice expressing the polyomavirus middle T oncogene. ***(A)*** *The expression of CD44 in the primary tumor is most prominent towards the edge of the tumor and decreases towards the center.* ***(B)*** *An adjacent section of the primary tumor shows that HA is most prominent towards the edge and decreases towards the center.*

Results and Discussion: As described in the previous progress report, the primary tumor present in the breast tissue was heterogeneous with respect to the expression of both CD44 and HA (Fig. 3 A and B). The amount of CD44 appeared to be highest at the edge of the primary tumor and decrease towards the center of the mass. A similar type of pattern was observed with the HA, with the highest concentration again towards the edge of the tumor. We believe that the distribution of HA and CD44 is a reflection of

the proliferation in the tumor mass. The cells that are actively dividing at the edge of the tumor express more CD44 and HA than the non-dividing cells in the center. If correct, then this would suggest that the turn over of HA is highest at the edge of the tumor.

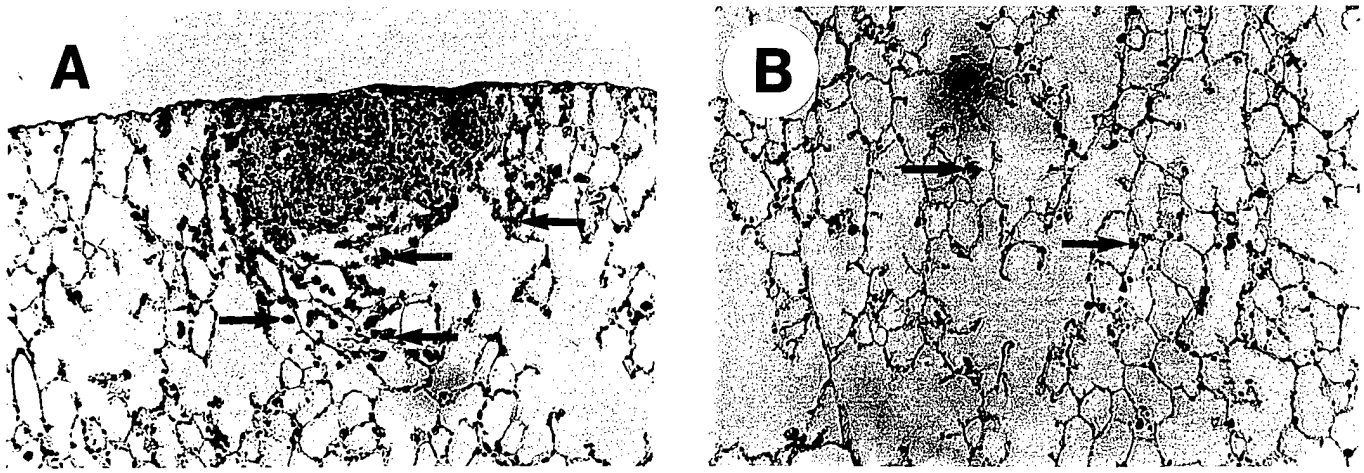

Fig. 4. The distribution of CD44 in a lung metastasis from a transgenic mouse. ***(A)*** *A section of the lung tissue shows that CD44 is expressed by the cells on the outer surface of the tumor mass but not by those in the interior. CD44 is also present on macrophages (indicated by the arrows) that surround the metastasis.* ***(B)*** *An adjacent region of normal lung tissue shows that the density of macrophage is greatly reduced (indicated by the arrows).*

We then examined the distribution of CD44 and HA in secondary tumors that were present in the lung tissue. Figure 4 A shows that the distribution of CD44 was similar to that of the original tumor, with positive staining on the cells located on the periphery and much less staining in the center of the tumor mass. In addition, a large number of macrophages that also stain for CD44 were apparent in the vicinity of the tumor (see arrows in Fig. 4 A), while adjacent sections of normal lung tissue contained far fewer macrophages (see Fig. 4 B). This is consistent with other studies showing that many tumors are associated with macrophages (34, 35). Interestingly, it appears that many of these macrophages are aggregated, perhaps as a response to elevated levels of HA.

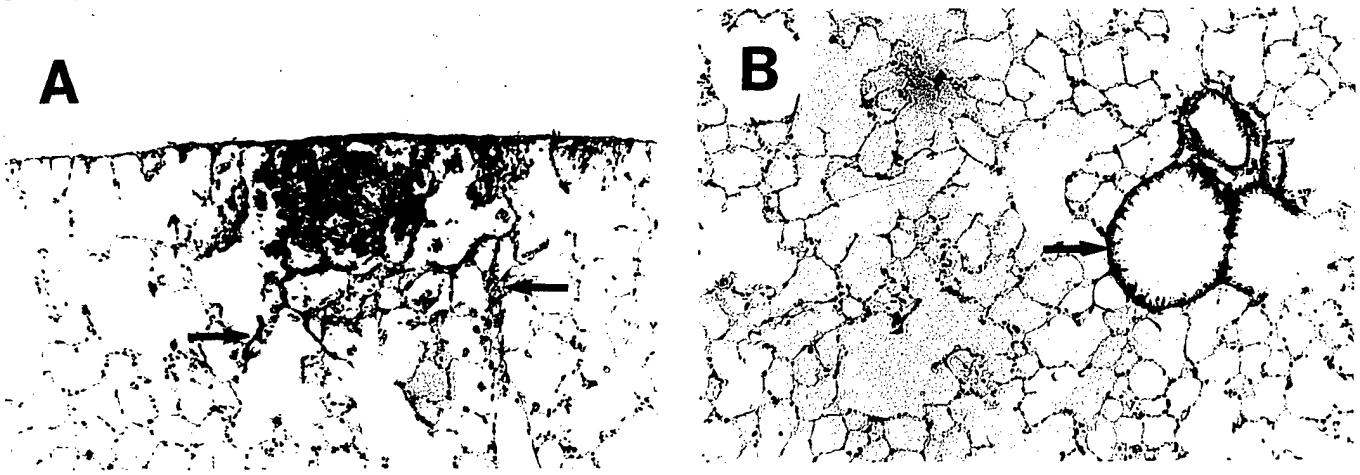

Fig. 5. The distribution of HA in a lung metastasis from a transgenic mouse. ***(A)*** *A section of the lungs shows that large amounts of HA are associated with the tumor mass. Much of this HA is located some distance from the tumor in the normal lung tissue (see arrows).* ***(B)*** *An adjacent section of normal lung tissue shows that HA is generally restricted to the adventitia of large blood vessels and air passage ways (see arrow).*

When the lung tissue was stained for HA, large amounts of HA were present in the alveolar tissue surrounding the tumor mass (Fig. 5. A). In contrast, adjacent normal lung tissue contained low levels of HA, most of which was present in the connective tissue surrounding the major blood vessels and air passage ways (Fig. 5. B). It appeared from Fig. 5 A that while some of the HA was associated directly with the tumor itself, much of the HA was located some distance away from the tumor mass in the surrounding normal tissue. The location of this HA suggested that that it was derived from the normal lung tissue perhaps as a result of a localized immune response. Along these lines, other studies have shown that an inflammatory response in the lungs results in increased levels of HA (36-38). It is also possible that this HA accounts for the observation described above that many of the macrophages in the vicinity of the tumor were clumped together, since we had previously shown that HA can induce these cells to aggregate by interacting with CD44 present on these cells (39).

This high levels of HA associated with the metastatic tumors in the lungs may have important implications. In particular, it suggested the possibility that this HA could be used to locate and target tumor metastases. This could be accomplished with the HA binding complex from cartilage that we had used previously as a histochemical stain for HA. This PG probe consists of a trypsin fragment of the aggrecan molecule along with an associated link protein that bind to hyaluronan with both high affinity and specificity (40). It may be possible to attach chemotherapeutic agents to this PG and inject this into individuals with tumors. Since the blood vessels that are associated with tumors are often leaky, the derivatized PG may be able to gain access to the HA that surrounds the tumors. Once this occurs, the PG along with the HA may be taken up by the tumor cells or associated macrophages, degraded in the lysosomal compartment and then the chemotherapeutic agent will released to effect the killing of the tumor cells.

The purpose of the new tasks was to test the feasibility of this approach. First, we determined if HA was associated with other cases of lung metastases, to determine if this phenomenon held true for other types of tumors as well (Task 5). Secondly, we tested the ability of biotinylated PG to target lung metastases (Task 6). And finally, in preliminary studies we have coupled the chemotherapeutic agent methotrexate to PG and tested its effectiveness on cultured cells (Task 7).

Recommendations: The observation concerning the association of HA with tumor metastasis should be investigated further (see Tasks 5, 6, and 7).

Task 4. Survey specimens of human breast tumor for the presence of CD44, HA and vascular endothelial cells: The purpose of this study was to determine if the expression of CD44 and HA was correlated with the distribution of blood vessels and if this could be used as a diagnostic indicator of tumor behavior. For this, we have made use of the Breast Cancer tumor bank which is one of the core facilitates of the Lombardi Cancer Center. Samples were selected from the tumor bank based upon the availability of specimens representing a spectrum of invasive tissue types including normal, ductal carcinoma *in situ*, and metastasis in the lymph nodes.

Previous results: As described in the previous progress report, when we examined normal breast tissue, we found that small amounts of CD44 were associated with the ductal cells and that HA was present in the stroma immediately surrounding the glandular epithelium, but was reduced or absent in regions located a short distance from the epithelium. In regions of invasive carcinoma and ductal carcinoma *in situ*, the tumor cells expressed high levels of CD44 and high levels of HA were associated with the surrounding stroma but was generally absent from the tumor mass. Finally, in the case of secondary tumors present in the lymph nodes, the expression of both CD44 and HA was variable. Taken together, these results indicate

that while CD44 was not consistently associated with metastatic tumors, it was associated with the presence or absence of HA in the tumor mass.

Staining for both HA and endothelial cells: During the past year, we have extended this study to examine the distribution of both HA blood vessels (endothelial cells). Paraffin sections of human breast cancer from the core facilitates of the Lombardi Cancer Center were simultaneously stained for both HA (red) and endothelial cells (blue). To accomplish this, the sections were first incubated with the b-PG probe for HA followed by peroxidase labeled streptavidin and the substrate 3-amino-9-ethylcarbazole that give a red reaction product. After extensive washing, the sections were incubated with rat anti-CD31, a marker for endothelial cells. The sections were then incubated with a biotinylated anti-rat IgG and peroxidase labeled streptavidin and the blue color was developed with HistoBlue. The chromogens were preserved with Crystal/Mount.

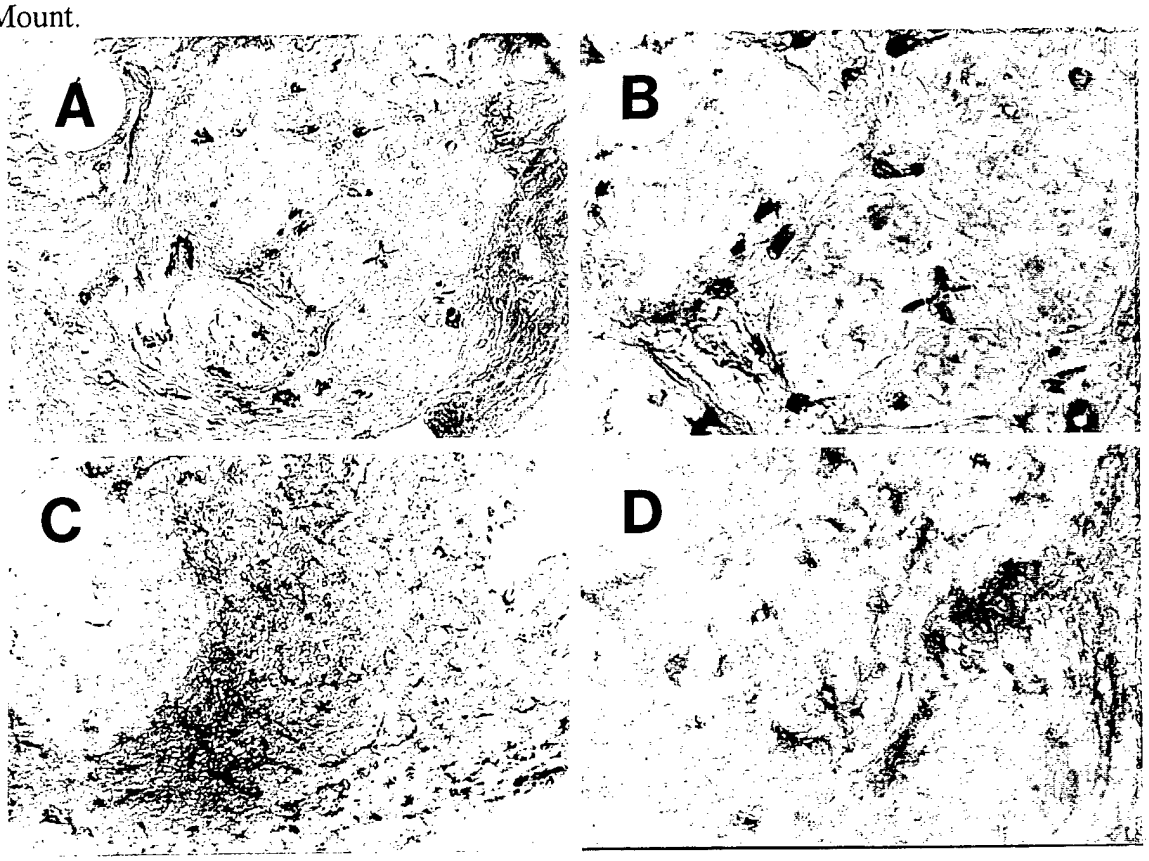

Fig. 6. The distribution of HA (red) and endothelial cells (blue) in two samples of human breast tumors. ***(A and B)*** *Low and high magnification views of one tumor shows that the endothelial cells are present in regions surrounding the tumor that are devoid of HA.* ***(C and D)*** *Low and high magnification views of a different tumor in which the endothelial cells coincide with the HA.*

Results and Discussion: The association between blood vessels and endothelial cells was quite variable. In the tumor sample shown in Figs. 6 A and B, blood vessels were present in the stroma surrounding the tumors that contain very little HA. However, in another tumor sample shown in Fig. 6 C and D, there are large number of capillaries in matrix that is rich in HA. Thus, there is not a consistent correlation between the expression of HA and the presence or absence of blood vessels. However, this does not necessarily disprove the hypothesis that fragments of HA can induce angiogenesis, since the HA may have been there originally when the blood vessels were initially formed and then subsequently lost. Thus, it is unclear if the distribution of HA has any predictive value in determining tumor angiogenesis.

Recommendations: While we have not found any consistent pattern between the distribution of HA and endothelial cells, we would like to confirm this by examining several more tumor sections.

Task 5: To examine various types of lung metastases for the expression of HA: As described above, the purpose of this set of experiments was to determine if metastases to the lungs are associated with increased levels of HA in the surrounding tissue. While this was clearly the case for the transgenic mouse described in Task 3, the question remained as to whether or not this is a generalized phenomenon for all forms of lung metastases. To answer this question, we examined the nude mice that had been given injections of human breast cancer cell lines as well as lung biopsies containing metastases from human patients suffering from breast cancer

Xenografts of human breast cancer cells in nude mice: In these experiments, approximately 10^6 human breast cancer cells (MDA-231 and Hs578T) were injected into the tail vein of nude mice and after one week, the mice were sacrificed and the lung tissue was collected. The lung tissue was embedded in paraffin, processed for histology and the resulting sections were then stained for HA using the b-PG probe in combination with peroxidase (red) and counterstained with hematoxylin (blue). As shown in Figs. 7 A and B, large amounts of HA staining were associated with the tumor nodules in the lungs. In these cases the HA appeared to be present in the normal tissue surround the tumor.

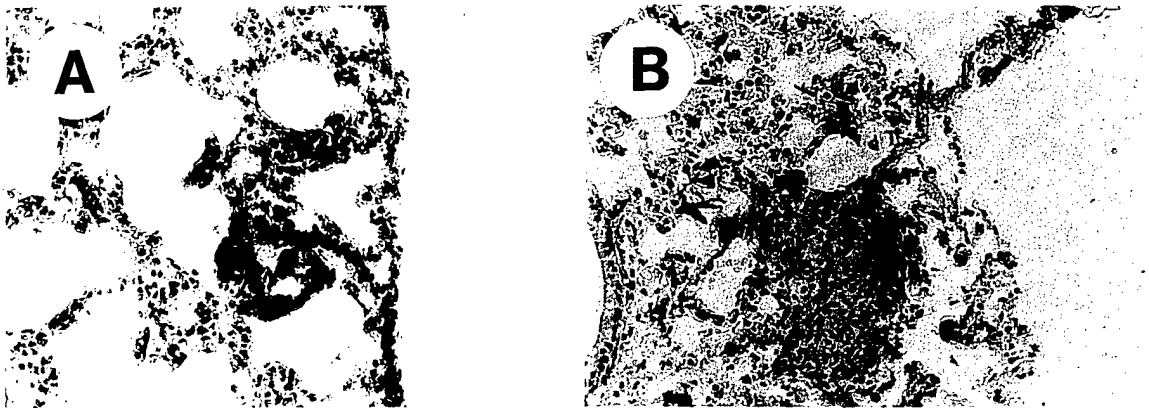

Fig. 7. The distribution of HA in the lungs of mice following the injection of human breast cancer cells. ***(A)*** *A nodule of MDA-231 cells in the lungs is surrounded by HA.* ***(B)*** *A nodule of Hs578T cells is also associated with HA.*

Perhaps the most dramatic illustration of this effect cames from experiments with the α-18 cell line which was derived from the human breast cancer cell line MCF-7 that had been transfected with expression vectors for both FGF and β-galactosidase. This last characteristic allows these cells to be detected by staining with X-gal. These cells were injected subcutaneously into nude mice, and the primary tumor metastasize to the lungs. Figures 8 A and B shows that large amounts of HA are associated with the stroma of the lungs surrounding these tumor cells which have been stained a light blue color with X-gal. In this case it is very apparent that the HA is present in the surrounding normal lung tissue as well as in the tumor cells.

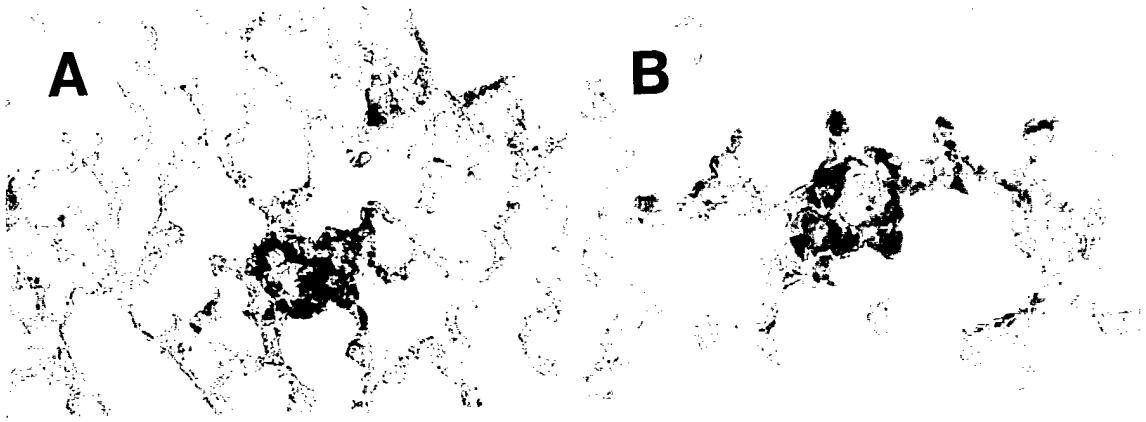

Fig. 8. The distribution of HA in the lung of mice with modules of α-18 cells. ***(A and B)*** *Low and high magnification views of the lungs showing tumor cells (light blue staining) surrounded by HA in the normal tissue.*

Lung metastases of human breast cancers: We have also examined the distribution of HA in biopsies of lung tissues from human patients suffering from breast cancer (from the core facilitates of the Lombardi Cancer Center). As illustrated in Figs. 9 A and B, in most, but not all cases, elevated levels of HA were present in the normal stroma surrounding the tumors. We believe that the more advanced the tumor is, the less likely that it will be associated with HA.

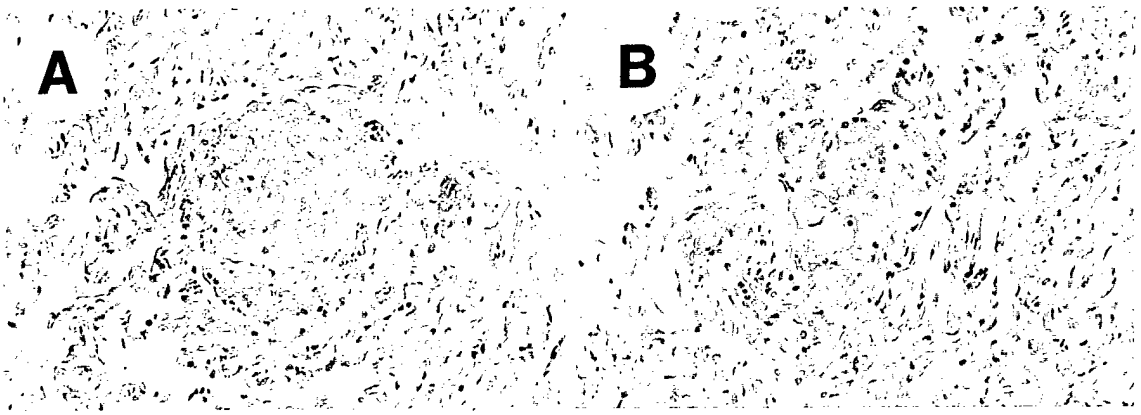

Fig. 9. Sections of lung tissue from patients suffering from metastatic breast cancer. ***(A and B)*** *Views of two different patients show that in these cases HA is present in and around tumor nodules.*

Results and Discussion: Similar results were obtained when we examined several different models of tumor metastasis. In most, but not all cases, we observed that large amounts of HA surrounded tumor nodules present in the lungs. In general, greater amounts of HA tended to be associated with newly formed tumors and much less with those that had been present longer. These results suggest that when tumor cells initially metastasize to the lungs, the initiate a response that induces the synthesis of HA.

At present the nature of the signal leading to the increase in HA production is not clear. One possibility is that the tumor cells initiates an immune response in the lung tissue that causes the normal lung cells to produce HA. This is consistent with previous studies that have found that inflammation in the lungs results in an increase in the production of HA (36-38). Along these lines, in preliminary studies we have found that dexamethasone treatment tends to down- regulate the HA associated with lung metastases, suggesting

that it is part of an immune response. Interesting, dexamethasone also stimulated the growth of tumors in the treated animals (data not shown).

Recommendations: If the tumor-associated HA is to be clinically useful, then it should also occur with other types of primary tumors (e.g. prostate and colon cancers) and on metastases present in other organs (e.g. the liver and lymph nodes). For this reason, we propose to extend this study to include other types of tumors and different sites of metastasis.

Task 6: To test the possibility of using PG to target lung metastases: The fact that HA is often associated with lung metastases suggested the possibility that it might be used to target these tumors using the HA binding proteins from cartilage. To test this possibility, we injected a biotinylated form of the cartilage proteoglycan (b-PG) into mice that had tumor metastases. We then examined the lungs of this mouse to determine if the b-PG had gained access to the HA associated with lung metastases.

Establishment of xenografts in nude mice: In these studies, we used the α-18 cell line, which is derived from MCF-7 cells that has been transfected with a bacterial *Lac-Z* gene. This allows the tumor to be easily located by staining with X-gal that gives a blue color. These cells have also been transfected with an expression vector for FGF-1 which allows them to metastasize to the lungs (41). Five million α-18 cells were injected into the mammary fat pats of 6-8 week-old nude mice and allowed to grow to form primary tumors of 1-2 gm and at the same time form spontaneous metastases to the lungs.

Preparation of biotinylated b-PG: The b-PG was prepared by extracting bovine nasal cartilage with 4 M guanidinium HCl to release the proteoglycan, dialyzing it against distilled water and finally dissolving it in 0.1 M Hepes, 0.1 M Na acetate. The extract was then briefly treated with trypsin followed by trypsin inhibitor to break it into small fragments (40). This extract was coupled to biotin with sulfosuccinimidyl 6-(biotinamido) hexanoate (40). And finally, the HA-binding fraction was isolated by affinity chromatography on HA coupled to Sepharose. The samples was then dialyzed against saline, and filter sterilized prior to use.

Injection of mice with b-PG: The mice bearing the α-18 xenografts were given i.v. injections of 200 μg of the b-PG. Twenty four hours after the injection, the mice were sacrificed, and the lung tissue was fixed in 3.7% formalin for 2 hours and then stained with X-gal. Following this, the tissue was embedded in paraffin and sectioned.

Histochemical localization of the b-PG: The sections were rehydrated, incubated with a solution of peroxidase coupled to streptavidin, followed by a substrate consisting of H_2O_2 and 3-amino-9-ethyl carbazole. The reaction was allowed to proceed for 30 minutes and the red chromogen was preserved with Crystal/mount.

Results and Discussion: In Fig. 10, a metastasis of the α-18 cell (blue stain) is closely associated with the red staining indicative of the b-PG. Thus, it would appear that under the conditions used, the b-PG can exit the blood vessels and interact with the HA associated with the tumor cells. It is also possible that some of this b-PG may have been internalized by the associated macrophages,

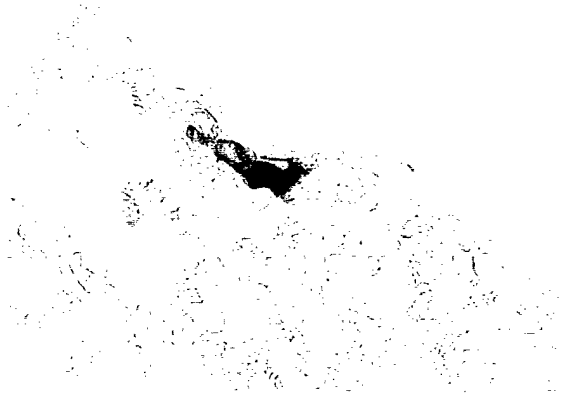

Fig. 10. The lung tissue of a mouse that had been given an injection of b-PG. The nodule of α-18 cells stains blue and the b-PG stains red.

however this was difficult to determine from the histological sections. It should be noted that the b-PG was not totally specific for the tumors since a small amount of staining was also detected in the HA-containing stroma surrounding the blood vessels and air passage ways (data not shown).

The results suggest that it may indeed be possible to use the PG complex to target lung metastases. The probe does appear to be preferentially associated with the α-18 cells present in the lungs. Thus, it may be possible to couple chemotherapeutic agents to the PG complex. It is our hope that in the vicinity of the metastasis, this complex this would be taken up by the tumor cells or by the associated macrophages. When the cells degrade the complex in the lysosomes, the chemotherapeutic agent would be released and kill the growing tumor cells. If the chemotherapeutic agent were cell-cycle dependent, then the tumor cells may be more susceptible than the associated macrophages which are post mitotic.

Recommendations: Clearly, the results of this study need to be confirmed and extended. The conditions such as the time after injection need to be optimized. In this preliminary study, we found that 24 hours was sufficient for the PG to penetrate to the target tissue. However, we do not know if longer periods of time may be better. Therefore, we will check the distribution of PG on days 1, 2 and 3 after injection. We will also examine other tissues to determine if the b-PG is localized in the liver and kidneys. If this is the case then we may co-inject chondroitin sulfate to block the binding of the PG to the liver endothelial cells. In this fashion, we will attempt to optimize the binding of the PG to the tumor cells relative to the normal tissue.

Task 7: To examine the effects of PG coupled to MTX on cultured tumor cells: As described above, we believe that drugs attached to the PG will bind to the tumor-associated HA and will be taken up by the tumor cells (or macrophages) using a CD44 dependent mechanism. In preliminary studies, we have examined the ability of PG coupled to a chemotherapeutic drug to kill tumor cells in culture. For this experiment, we used methotrexate (MTX), which is widely used as a chemotherapy drug (42, 43). MTX is considered to be a cell-cycle specific agent that is most effective against rapidly proliferating tumors such as leukemia and to a lesser extent carcinomas of the breast and testis (43). Structurally MTX closely resembles folic acid and acts as an agonist of this cofactor. It functions by binding tightly to dihydrofolate reductase, an enzyme that is critically involved in the synthesis of DNA, RNA and proteins. While MTX is not entirely specific for cells undergoing cell division, it is our hope that the conjugate of MTX-PG will not be toxic to macrophages which are post-mitotic. In addition, we also hope that the MTX-PG will be less toxic to the cells of the bone marrow, skin and gastrointestinal track than MTX by itself.

Preparation of MTX conjugated PG: The conjugation was carried out according to the methods described by Kulkarni (44) which involves two steps. In the first step, an active ester intermediate of MTX was prepared. In the second step, this MTX derivative was coupled to PG using N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride. Following the reaction the MTX-PG in the supernatant was purified by HA affinity chromatography (40). The amount of MTX coupled to the protein was determined from the OD_{529}.

Cytotoxicity assay: For this experiment, the rat fibrosarcoma (RFS) cell line which expresses a large amount of HA and CD44 was plated into 96 well plate (2,000 cells/well) and after 8 hours the medium was changed to one containing the test substances. In this assay, equivalent amounts (10 μM final concentration) of coupled MTX and MTX alone were added based on the OD_{529}. One day later, ^{3}H-thymidine was added to the wells and the cultures were allowed to grow for an additional 8 hours. The cells were harvested and the amount of incorporated ^{3}H-thymidine was determined with a β-counter.

Results and Discussion: As shown in Fig. 11, the complex of MTX-PG significantly inhibited the proliferation of the tumor cells. Indeed, the activity of the MTX-PG was approximately the same as that of equivalent amount of MTX by itself. In general, the when a drug has been coupled to a proteins such as an antibody, it looses its toxicity (45, 46). This is not surprising since the complex must be internalized and degraded by the tumor cell before the coupled drug will be released and exert its activity. Thus, the fact that the complex of MTX-PG is active is a very encouraging.

Recommendations: We have just started these experiments, and clearly they need to be confirmed and extended. First, we need to test the human breast cancer cell lines (for this initial study we used RFS cells because they have a large amount of HA associated with their surfaces). Second, we need to do dose response curves with the MTX-PG complex. And third, we need to determine if the effect can be blocked by antibodies to CD44, hyaluronidase and an excess of unlabeled PG. If the uptake is dependent upon CD44, then these agents should block the effect. Once, we have optimized the conditions for killing tumor cells in culture, we can then apply this agent to experimental models of cancer.

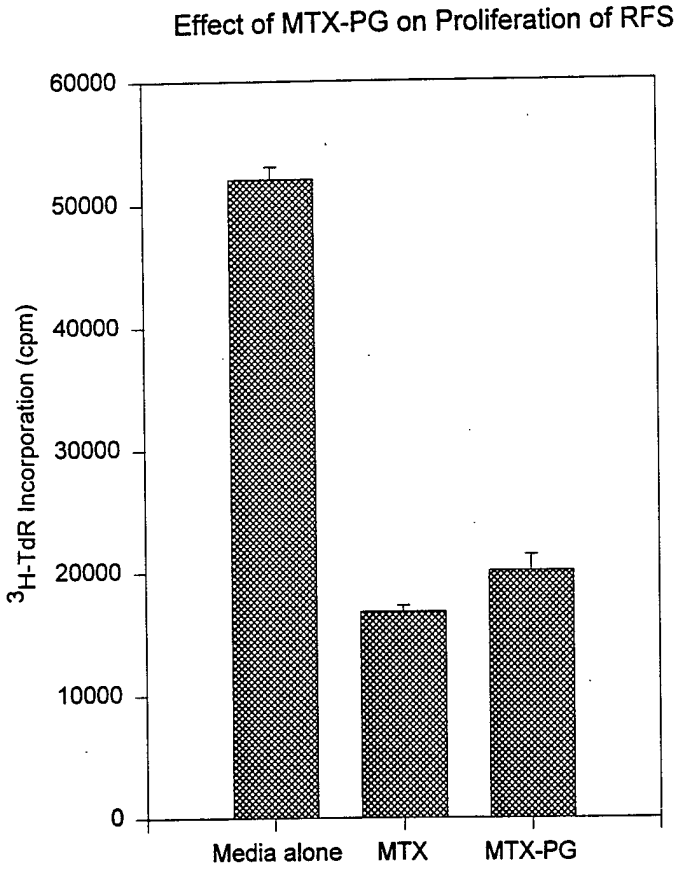

Fig. 11. The effect of MTX and MTX-PG on the proliferation of RFS cells. Approximately 10 μM MTX was added to each culture.

CONCLUSIONS:

Implication of Completed Research (both old and new):

1) Both primary and secondary tumors of breast cancer are heterogeneous with respect to the expression of CD44. Thus, there does not appear to be a close correlation between the expression of CD44 and the formation of metastases.
2) In general, there is an inverse correlation between the expression of CD44 and the presence of HA. Presumably, this is due to the fact that CD44 allows cells to take up and degrade HA. However, exceptions to this were noted in several cases. It is possible that in these regions, the extent of HA synthesis is so great, that the CD44 mediated degradation is not sufficient to remove all of it.
3) The presence of HA does not appear to be directly correlated with the distribution of endothelial cells (blood vessels) in biopsies of human breast cancer.
4) Large amounts of HA are associated with tumors that have metastasize to the lungs. This phenomenon may be useful for targeting agents to the tumors.
5) When b-PG is injected into mice, it can become preferentially associated with tumor metastases present in the lungs.
6) A complex consisting of MTX coupled to PG is active in blocking the proliferation of tumor cells in culture.

Recommended Changes:

1) In Task 1, new cells lines should be used for transfection with the CD44 expression vectors.
2) The feasibility of using derivatives of PG (and perhaps HA) to target tumors should be further investigated. In other words, the new Tasks 6 and 7 should be expanded.

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ACRONYMS AND SYMBOL DEFINITIONS

[^{3}H]HA	Tritium labeled hyaluronan.
b-Hermes-1	Biotinylated form of the hermes-1 monoclonal antibody - used for the localization of human CD44.
b-KM-201 mAb	Biotinylated form of the KM-201 mAB
b-PG	Biotinylated proteoglycan - used as specific staining probe for hyaluronan.
CD44	Cluster of determination (differentiation) - same as the hyaluronan receptor or binding site.
CMF-PBS	Calcium and magnesium free phosphate buffered saline.
DMEM	Dulbecco's modifed Eagle's medium
HA	Hyaluronan.
HAase	Hyaluronidase (either testicular or *Streptomyces*)
Hermes-1 mAb	Monoclonal antibody against human CD44 - blocks the interaction with hyaluronan.
K-3 mAb	Monoclonal antibody against hamster CD44 - blocks the interaction with hyaluronan.
KM-201 mAb	KM-201 monoclonal antibody directed against mouse CD44 - blocks the interaction with hyaluronan.
mAb	Monoclonal antibody.
MTX	Methotrexate, a cell-cycle dependent chemotherapeutic agent.
MTX-PG	A derivative of MTX coupled to the PG complex.
PG	A complex of a trypsin fragment of cartilage proteoglycan and link protein that binds to HA with high affinity and specificity.
RFS	Rat fibrosarcoma cell line that express large amounts of HA on its surface.
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
SV-3T3	Simian virus 40 transformed mouse 3T3 cells (Swiss mouse).