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The goal of this research project is to examine the roles of hyaluronan and its cell surface receptor, CD44, in tumor progression. In task 1, we have transfected a series of human breast cancer cell lines with a CD44 expression vector. These transfected cells can both bind and degrade hyaluronan, indicating that the CD44 is biologically functional. These cell have been injected into nude and once the tumors have grown they will be examined for both hyaluronan and endothelial cells. In task 3, we have examined primary and secondary tumors in mice that are transgenic for the polyomavirus middle T oncogene. While primary tumors were heterogeneous with respect to the expression of CD44, secondary tumors in the lungs did not express this molecule, suggesting that CD44 is not required for metastasis. More importantly, the secondary tumors were associated with large amounts of hyaluronan, which may be useful for targeting these tumors. In task 4, we have examined sections of human breast cancer. While the primary tumors expressed significant amounts of CD44, secondary tumors in the lymph nodes showed a considerable variation with respect to the expression of CD44. This expression of CD44 was inversely associated with hyaluronan.

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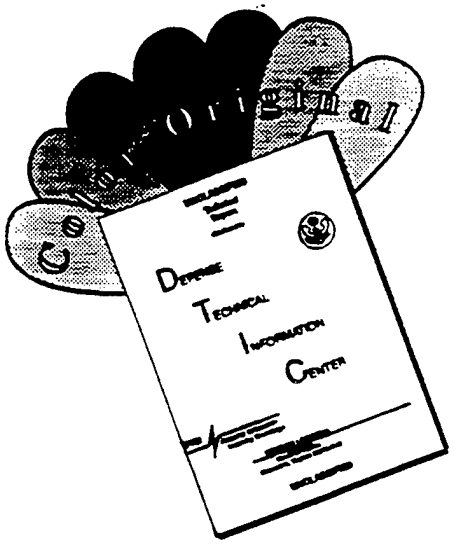

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INTRODUCTION:

Nature of the Problem:

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

The working hypothesis of the present application is that this CD44-mediated degradation of hyaluronan 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 hyaluronan. Thirdly, the fragments of hyaluronan produced in the process of degradation have angiogenic properties leading to increased vascularization. And fourthly, large amounts of hyaluronan surround many types of blood vessels, and the degradation of this hyaluronan 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

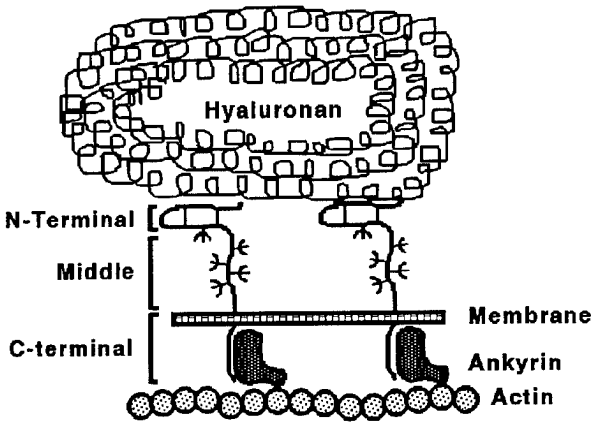

Fig. 1. Model of CD44 and its interactions with the cytoskeleton and HA.

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 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.

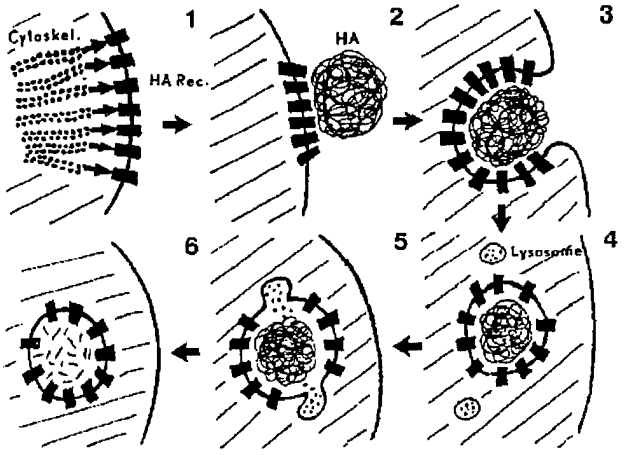

Fig. 2. Model of HA degradation. (1) Initially molecules of CD44 are clustered on the cell surface through their interaction with the cytoskeleton. (2) A number of molecules of CD44 bind simultaneously to a molecule of HA. (3 and 4) The HA is endocytosed into a vesicle. (5) Lysosomes fuse with the vesicle.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

The working hypothesis of this proposal is 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.

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 select cells that stably express this protein. Both CD44 positive and negative cells will be injected into nude mice and allowed to grow. The resulting xenografts will be surgically removed and examined histologically 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. In a similar experiment, the osmotic pump will be filled with oligosaccharide fragments of HA or HAase, which should enhance vascularization of tumor cells that do not express CD44 if the working hypothesis is correct.

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. This suggests that while primary tumors may be mixed with respect to the expression of CD44, the secondary tumors (i.e. metastases) will be selected from the CD44 positive sub population. To examine 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 and HA in evaluating its metastatic potential, we will examine specimens of human breast cancer, which are available from the tumor bank of the Lombardi Cancer Center. The specimens in this collection will have been classified by various criteria including survival of the donor. In this pilot study, we will analyze the presence of CD44 and HA in subsets of these tumor specimens with respect to metastatic versus non metastatic (node positive vs. node negative) and short versus long survival term survival of the donor. If this pilot experiment shows a good correlation between these parameters then we will expand this study to include a greater number of samples.

BODY

During the second year of this project, we have used the reagents prepared in the first year to carry out the proposed studies. The progress that has been make on each of the tasks will be described in the following sections.

Task 1: Examine the effect of CD44 expression of the vascularization of tumors: The purpose of this study is to test our working hypothesis that CD44 enhances the vascularization of tumors. To accomplish this, we have transfected human breast cancer cell lines (MCF-7 and ML-20) with a CD44 expression vector. These cells will then be grown in nude mice, and the resulting xenografts will be analyzed histochemically for endothelial cells.

Previous results: As described in the previous progress report, we transfected the ZR-751 clone of human breast cancer cells with an expression vector for human CD44. After expending a large amount of effort, we found that neither the control nor the CD44 transfected ZR-751 cells were able to grow in nude mice. Apparently the ZR-751 cells had changed their character as a result of extensive culturing *in vitro*. In view of this problem, we altered our original protocol and have begun to use the MCF-7 cell line which also express low levels of CD44 and retains the capacity to grow in nude nice. In addition, we have also used the ML-20 cell line which is derived from MCF-7 cells and expresses β-galactosidase as a marker for metastasis. As will be described in the following sections, we have isolated a number of CD44 positive cell lines which have been injected into nude mice. At present, we are still waiting for the tumors to grow so that they can be examined histochemically.

Preparation of the Plasmid Vector: In initial studies, we found that our original CD44 vector was defective in that the transfected CD44 was unable to bind HA. For this reason, we have selected another vector that gives positive results. The cDNA for the full length mouse CD44 was inserted into the *Xba I* and *Hind III* sites in the mammalian expression vector pRc/CMV (In Vitrogen, San Deigo, CA). This plasmid (pRc/CMV-CD44) as well as an empty control vector (pRc/CMV-control) were amplified in DH5α cells and purified using the Wizard Midiprep kit from Promega, WI. The plasmids were not linearized prior to transfection into the mammalian cells.

Transfection of Cultured Cells: The human breast cancer cell lines MCF-7 and ML-20 were grown in 10% fetal calf serum, 90% DMEM to a density of approximately 10^6 cells per 100 mm plate. The medium was changed and four hours later 30 μg/plate of the plasmid DNA adsorbed to calcium phosphate was added to the medium (made by mixing the plasmid DNA, $CaCl_2$ and phosphate buffered saline). After 24 hours, the cells were trypsinized and subcultured from one to five plates. After an additional 24 hours, 800 to 1,000 μg/ml of G-418 sulfate (geneticin) was added to each of the plates. The cells were allowed to grow for an additional 2 weeks, and the clones of surviving cells were picked up with pieces of filter paper soaked with trypsin and transferred to 24 well plates for expansion. Approximately 24 clones for each condition were selected for analysis.

Screening of Clones for CD44: Initial screening of the clones was carried out using dot blot analysis. For this, the cells were extracted in DOC buffer (0.1% Na deoxycholate, 0.5 M NaCl, 0.02 M Tris pH 8.0) and applied to a sheet of nitrocellulose (Immobilon-NC, Millipore, MA) using a dot blot apparatus. The sheet of nitrocellulose was blocked in 5% non-fat milk and incubated with a solution of the KM-201 mAb which recognizes mouse CD44 (10 μg/ml in 10% calf serum, 90% phosphate buffered saline). This was followed sequentially by incubations with peroxidase labeled anti-mouse IgG and a chemiluminescent substrate (ECL substrate kit, NEN/DuPont). Figure 3 of the exposed X-ray film shows that a number of the ML-20

(A) and MCF-7 (B) clones transfected with the pRc/CMV-CD44 plasmid expressed CD44 (lanes 4-12) while none of those transfected with the pRc/CMV-control (lanes 1-3) expressed this protein.

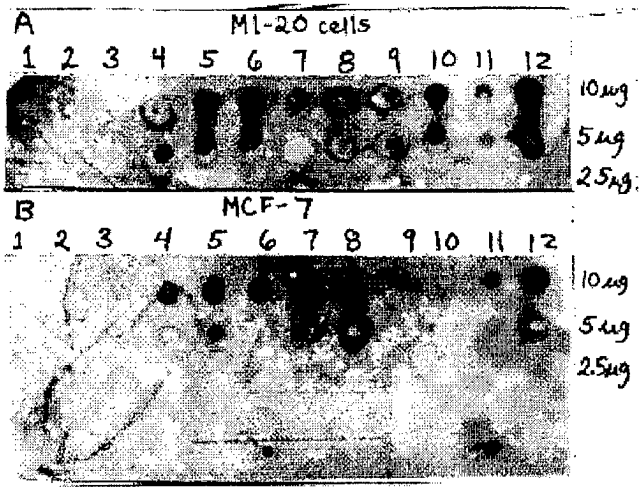

Fig. 3. Dot blot analysis of clones (A, ML-20; B. MCF-7) transfected with both control (lanes 1-3) and CD44 (lanes 4-12) expression vectors. Following transfection, geneticin-resistant cell clones were selected. The cells were solubilized in detergent and applied to a sheet of nitrocellulose using a dot blot apparatus. The sheet was then immunostained for mouse CD44 with the KM-201 mAb. While all of the clones transfected with the control vector were negative (lanes 1-3), varying amounts of immunoreactivity were apparent in those transfected with the CD44 expression vector (lanes 4-12).

Western Blotting: The clones that were assessed to be positive by the dot blot technique were further analyzed by Western blotting. The clones were extracted in Laemmli sample buffer (without reducing agents), and electrophoresed on a 7.5% SDS polyacrylamide gel. Following electrophoresis, the proteins were transferred to a sheet of nitrocellulose (0.9 amps, 30 min) which was then blocked with 5% non-fat milk. The sheet of nitrocellulose was incubated with the KM-201 mAb to the mouse CD44 and further processed as described above. Figure 4 shows that the bulk of the immunostaining was in a band that runs at approximately 85 kDa as expected for mouse CD44 (lanes 3 through 9). The clones transfected with the control vector were negative (lanes 1 and 2). These results confirm the dot blot data.

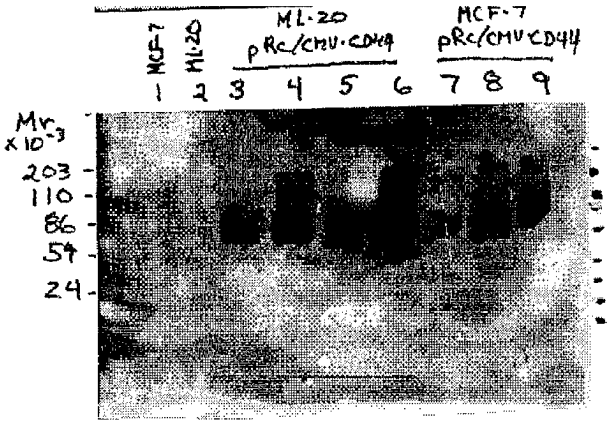

Fig. 4. Western blot analysis of ML-20 and MCF-7 cells transfected with control (lanes 1 and 2) and CD44 expression vectors (lanes 3 through 7). Detergent extracts of the cells were electrophoresised on an SDS- polyacrylamide gel, transferred to a sheet of nitrocellulose which was then stained with the KM-201 antibody to mouse CD44. The cells transfected with the control vectors (lanes 1 and 2) were negative, while those transfected with the CD44 expression vector (lanes 3 through 9) showed an immunopositive band at approximately 85 kDa, which is consistent with mouse CD44.

HA Binding Activity: The selected clones were then analyzed for their ability to bind isotopically labeled HA to measure of the functional activity of the transfected CD44. In this assay, the cells were extracted in DOC buffer and then mixed with set amounts of [^{3}H] HA. The background level of binding was determined by added a large excess of non-labeled HA to some of the samples. After a short incubation, an equal volume of $(NH_4)_2SO_4$ was added to the samples along with a small amount of non-fat milk to act as a carrier. The $(NH_4)_2SO_4$ precipitates the proteins including the CD44 as well as any [^{3}H] HA that was bound to it. However, free [^{3}H] HA was left in solution. The samples were centrifuged and the precipitate was carefully washed with 50% saturated $(NH_4)_2SO_4$. The amount of label present in the precipitates were determined by scintillation counting. As shown in Fig. 5, the cell lines that were positive for CD44 by dot

and Western blotting were also able to bind significant amounts of [^{3}H] HA. In contrast the clones transfected with the control vector bound less [^{3}H] HA. These results indicate that the transfected CD44 was effective in binding HA.

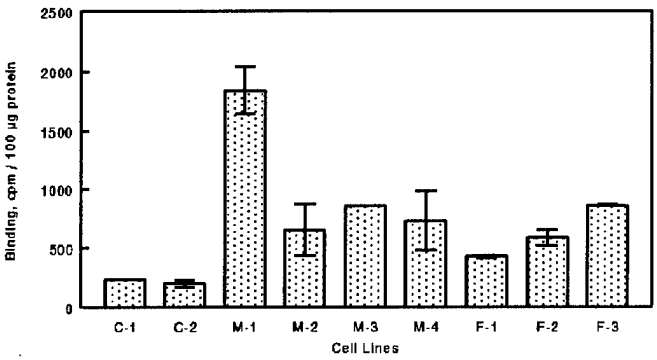

Fig. 5. HA binding activity of clones transfected with control and CD44 expression vectors. Detergent extracts of the clones were mixed with [^{3}H]HA, to which was added an equal volume of $(NH_4)_2SO_4$ to precipitate the CD44 and any bound [^{3}H]HA. The amount of label present in the precipitate was then determined by scintillation counting. In each case the background level of binding which was determined by adding a large excess of cold HA, has been subtracted. C-1, MCF-7 control (vector alone); C-2, ML-20 control; M-1 through M-4, ML-20 CD44 transfected clones; F-1 through F-3, MCF-7 CD44 transfected clones.

Degradation Assay: The final functional assay was to determine if the CD44 allowed these cells to degrade HA. In this assay, the cells were cultured in the presence of [^{3}H] HA (23) for 48 hours, subjected to freeze-thawing to lyse the cells and digested with pronase E. The resulting digests were applied to the tops of Centricon 30 Microconcentrators (Millipore, MA) and centrifuged. The fragments of [^{3}H]HA that passed through the size specific membrane were collected and analyzed by scintillation counting. As shown in Fig. 6, the transfected cells that expressed the CD44 were also able to degrade significantly greater amounts of the HA than the control cells. Furthermore, this degradation was inhibited by the addition of both cold HA and by the blocking monoclonal antibody KM-201, but not by a nonspecific mouse IgG (see Fig. 6). These results suggest the CD44 is required for the degradation of the HA.

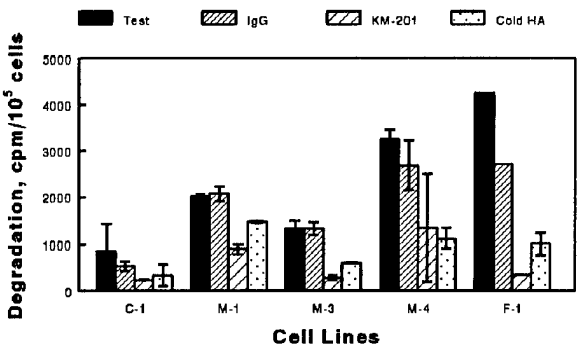

Fig. 6. The degradation of [^{3}H]HA by cells transfected with a control and CD44 expression vectors. The cells were cultured in the presence of [^{3}H]HA for 2 days, and then the amount of degraded HA was assessed by amount of fragments able to pass through a size specific membrane. The cell lines transfected with the CD44 expression vector degraded significantly greater amounts of [^{3}H]HA than those transfected with the control vector (C-1). While the extent of degradation was not influenced by non-specific IgG, it was inhibited by the KM-201 mAb to CD44. Furthermore, the addition of a large excess of non-labeled HA also blocked the degradation. C-1, MCF-7 control (vector alone); M-1 through M-3, ML-20 CD44 transfected clones; F-1, MCF-7 CD44 transfected clone.

Furthermore, the degradation of [^{3}H]HA by each of the clones was inhibited by the addition of NH_4Cl to the medium of the cultured cells (see Fig. 7). Ammonium chloride can prevent the acidification of

lysosomes so that the action of acid hydrolases is inhibited. Taken together, these results suggest that the degradation of [^{3}H]HA is a multi-step process in which the HA is first bound to the cell surface by CD44, it is then internalized and finally brought to a lysosomal compartment where it undergoes hydrolysis.

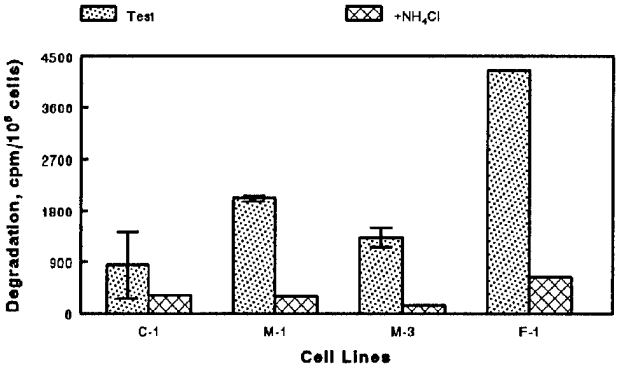

Fig. 7. The effect of NH_4Cl on the degradation of [^{3}H]HA by the transfected clones. Ammonium chloride (10 mM) was added to the medium of the cell during the degradation assay which was carried out as described in Fig. 6. This agent blocked most of the degradation of [^{3}H]HA by each of the cell lines suggesting that acidification of the lysosomal compartments is required for this process.

In Vivo Characterization: The cells transfected with the control and CD44 expression vectors were then injected into nude mice. For this, the cells were cultured in 10% fetal calf serum, 90% DMEM in 150 mm dishes and when the plates were at 90% confluence, the cells were harvested with 10 mM EDTA in PBS. To avoid clonal variations, a number of different clones from each transfection were examined. Approximately 10^7 cells were injected per site into the mammary fat pads of nude mice (four to six week old from NCI under contract with the Georgetown Animal Facility). Each cell line was injected into 6 sites with medium alone. To increase the rate of tumor formation, the cell were mixed with Matrixgel (Collaborative Research, MA) and injected into another 6 sites. At this point (i.e., one week after the initial injection) tumors were apparent for each of the cell lines, however, there were no obvious differences in the tumor take rate or their size.

After the tumors have grown some what larger (approximately 2 to 4 more weeks), the mice will be given an i.p. injection of pentobarbital (Nembutal, 90 mg/kg body), and then sacrificed by exsanguination. The resulting tumor mass will be fixed and processed for histology. The sections will be stained for 1) CD44 using either the KM-201 mAb; 2) HA using the b-PG binding probe: and 3) endothelial cells, using a monoclonal antibody to mouse PECAM-1 (Pharmingen). This latter agent was found to be more specific for mouse endothelial cells than the lectin *Dolichos Biflorus agglutinin* that was initially proposed (preliminary studies). From the results of this staining, we will quantitate the number of endothelial cells by counting random fields at the periphery of the xenografts. If our working hypothesis is correct, then a greater number of endothelial cells and blood vessels will be associated with the xenografts expressing CD44, than with those that do not. If no such association is observed then we will reassess the initial working hypothesis.

Task 2. Determine the effects of various agents on the vascularization of tumors expressing CD44: The purpose of this set of experiments is to determine if xenograft vascularization can be blocked by antibodies to CD44 or enhanced by fragments of HA or HAase. We will begin these experiments once an appropriate cell line has been identified in Task 1. At present, we have prepared the following reagents to be tested:

KM-201 mAb and Fab fragments: The KM-201 is a rat mAb directed against mouse CD44 which blocks its ability to interact with HA (3). The ascites fluid from the KM-201 hybridoma was prepared by a commercial laboratory (Bioproducts for Science, Indianapolis, IN). The KM-201 mAb was purified from the ascites fluid using the Econo-Pac serum IgG purification kit from Bio-Rad. IgG was also purified from rat serum to serve as a control. The Fab fragments were prepared by digesting the purified mAb with immobilized papain according to the method provided by the Pierce Chemical Company. The Fab fragments were separated from the Fc fragments by chromatography and found to be pure by SDS-PAGE analysis (40). The control for this agent consists of Fab fragments of IgG from whole mouse serum.

Oligosaccharide Fragments of HA: The oligosaccharides were prepared by a limited digestion of a highly purified preparation of HA (Healon, Pharmacia) with testicular HAase (41). The enzymatic digestion was terminated by placing the sample in a boiling water bath for 10 min. Molecular sieve analysis of the product indicates that it has a molecular weight in the range of 10 to 100 kDa.

HAase: Highly purified preparations of both testicular and *Streptomyces* HAase have been purchased from commercial sources.

The agents described above will be first analyzed for endotoxin and if they are negative, they will be placed into small osmotic pumps. These pumps will then be implanted into nude mice along with CD44 transfected MCF-7 or ML-20 cells. After 8 weeks, the mice will be sacrificed the tumors will be examined histologically for HA and endothelial cells as described above. If our working hypothesis is correct then we would anticipate that tumor vascularization will be inhibited by Hermes-1 and stimulated by oligosaccharide fragments of HA and HAase.

Task 3. Examine the expression of CD44 in primary and secondary tumors of transgenic mice: The purpose of this set of experiments is to compare primary versus secondary tumors with respect to the expression of CD44 and HA. In initial experiments, we have 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 (45). This transgenic strain of mice forms multifocal mammary adenocarcinomas that metastasize to the lungs at a high frequency.

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 (34) 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 (35). The sections were then counterstained with Meyer's hemotoxylin that gives a blue color. The chromogens were preserved with Crystal/mount and then coversliped.

The results of this staining procedure are shown in Fig. 8. The primary tumor mass was found to be heterogeneous with respect to the expression of CD44. In general, the leading edge of the primary tumor was positive for CD44, while more internal tissue was negative (Fig. 8 A). However, necrotic regions in the center of the primary tumor also expressed CD44 (data not shown). The distribution of HA in the primary tumors was similar to that of the CD44, being high on the leading edge and greatly diminished or absent from more central regions of the tumor mass (Fig. 8 B), except in regions of necrosis where the

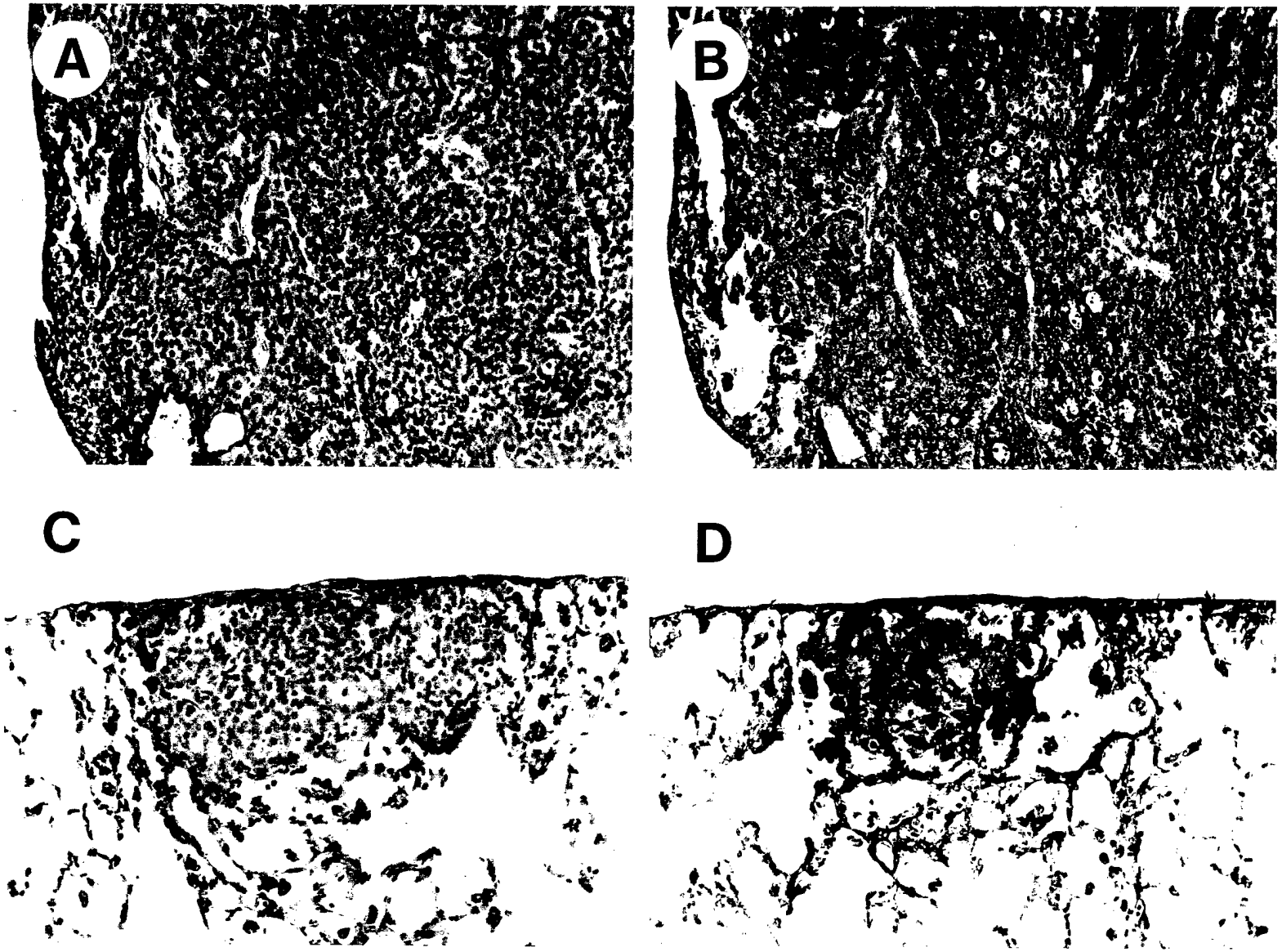

Fig. 8. Distribution of CD44 and HA in primary and secondary tumors from transgenic mice expressing the polyomavirus middle T oncogene. (A) The expression of CD44 in the primary tumor is most prominent towards the edge of the tumor and decreases towards the center (it increases again in necrotic regions that are not shown). (B) An adjacent section of the primary tumor shows that HA is most prominent towards the edge and decreases towards the center (it increases in necrotic regions that are not shown). (C) A secondary tumor present in the lungs does not express significant amounts of CD44. The positive staining cells surrounding the tumor mass correspond to macrophages. (D) An adjacent section of the secondary tumor shows that large amounts of HA are associated with the tumor mass.

levels increased again (data not shown). This pattern was somewhat surprising to us, since we expected to see an inverse correlation between the expression of CD44 and HA (since the CD44 has been implicated in the removal of HA). The most likely explanation is that the amount of HA present is a function of both its synthesis and degradation. At the edge of the tumor, there may be an increase in both the synthesis and degradation of the HA.

We then examined the distribution of CD44 and HA in secondary tumors that were present in the lungs. As shown in Fig. 8 C, the secondary tumor did not express significant amounts of CD44. Thus, CD44 does not impart any particular survival advantage to secondary tumors as we had initially predicted. It should be noted that the large number of CD44 positive cells that surround the tumor mass (see Fig. 8 C) correspond to pulmonary macrophages.

More significantly, Fig. 8 D shows that large amounts of HA are associated with the secondary tumors in the lungs. This staining pattern was quite striking in that HA was generally absent from the lung tissue except in the stroma around large blood vessels and air passage ways. At present, we do not know whether this HA is derived from the tumor itself or from the normal lung tissue. Similarly, we do not know if a similar pattern of HA is associated with tumors that have metastasized to other organs such as the liver or brain.

This observation concerning the association of HA with these secondary tumors may be clinically important. It may be possible to target this tumor-associate HA with chemotheraputic agents that can block the growth of the tumor cells. This possibility is presently being investigated.

Task 4. Survey specimens of human breast tumor for the presence of CD44, HA and vascular endothelial cells: In this final task, we propose to carry out a pilot study to determine if the expression of CD44 can be used as a diagnostic indicator of tumor behavior. For this study, 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. In initial studies, samples from two different individuals with breast cancer were examined.

For the staining of CD44 we used the BMS113 mouse monoclonal antibody from Biosource International (CA) which binds to the standard form of human CD44 in paraffin-embedded tissue. However, it should be noted that this antibody also stains the connective tissue stroma, which complicates the interpretation of the staining pattern. (At present, we do not know if this stromal staining is non-specific or is to the presence of CD44 like molecules in the stroma.) The sections were rehydrated and incubated with a 1:50 dilution of the antibody in 10% calf serum, 90% PBS for one hour. This was followed by peroxidase labeled anti-mouse IgG diluted 1:250 in 10% calf serum, 90% PBS. The immunoreactivity was developed with a peroxidase substrate which gives a red reaction product and counterstained with Meyer's hematoxylin that gives a blue color (see methods in Task 3). For staining for HA, we used the b-PG probe that was derived from cartilage proteoglycan (see methods in Task 3).

The pattern of CD44 and HA staining in tissues from the two different individual are shown in Figs. 9 and 10. The left hand panels show the staining of CD44 while the right hand panels show the staining of HA in equivalent regions. In the case of the normal breast tissue (Figs. 9 A and 10 A) small amounts of CD44 were associated with the normal ductal cells (please note that the antibody also stains the stroma). In this

normal tissue (Figs. 9 B and 10 B), 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 the regions of invasive carcinoma (Fig. 9 C) and ductal carcinoma *in situ* (Fig. 10 C), the tumor cells expressed high levels of CD44, and high levels of HA were associated with the surrounding stroma but was absent from the tumor mass itself. (Figs. 9 D and 10 D).

Finally, in the case of secondary tumors present in the lymph nodes, the expression of both CD44 and HA was variable. For the metastatic tumor shown in Figs. 9 E and F, the majority of the cells expressed CD44 and the level of HA was low. In contrast, for the tumor shown in Figs 10 E and F, the tumor cells express little or no CD44, while significant levels of HA were present within the tumor mass. Taken together, these results indicate that while CD44 is not consistently associated with metastatic tumors, it is associated with the presence or absence of HA in the tumor mass.

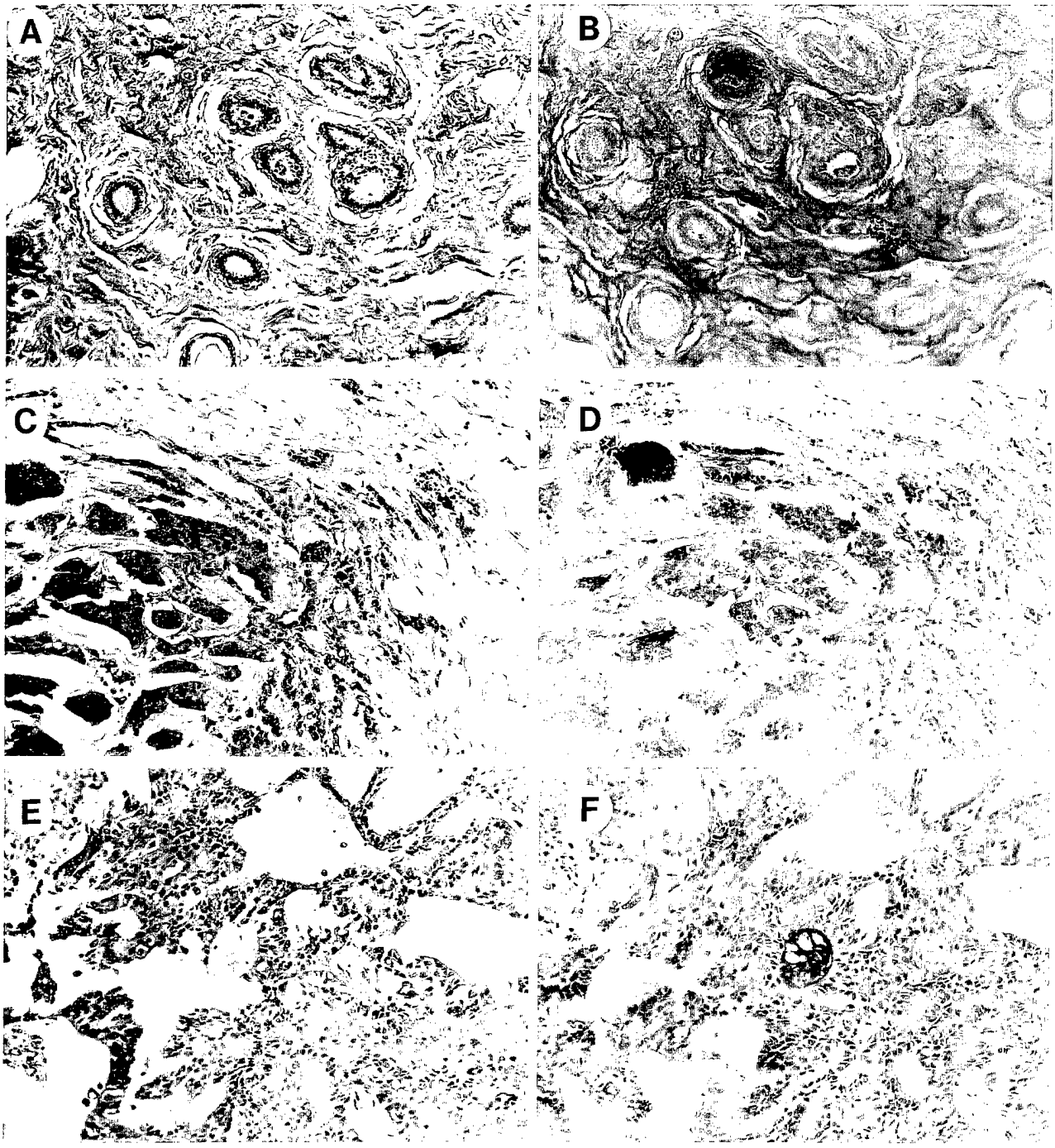

Fig. 9. The distribution of CD44 and HA in paraffin sections of tissue from a patient with breast cancer. The CD44 was stained using the BMS113 mAb and the HA was stained with b-PG (red color). The sections were counterstained with hematoxylin (blue color). (A) Normal breast epithelium showes relatively low levels of CD44 (note: the staining in the stroma should be ignored). (B) HA is present in the stroma surrounding the normal breast epithelium. (C) This invasive carcinoma expresses higher levels of CD44 than the normal tissue. (D) An adjacent section shows that HA is present in the surrounding stroma, but absent from the tumor mass. (E) A region of a lymph node shows that the metastasized tumor cells express CD44. (F) An adjacent region shows that the tumor mass contains very little HA (except in small isolated nodules).

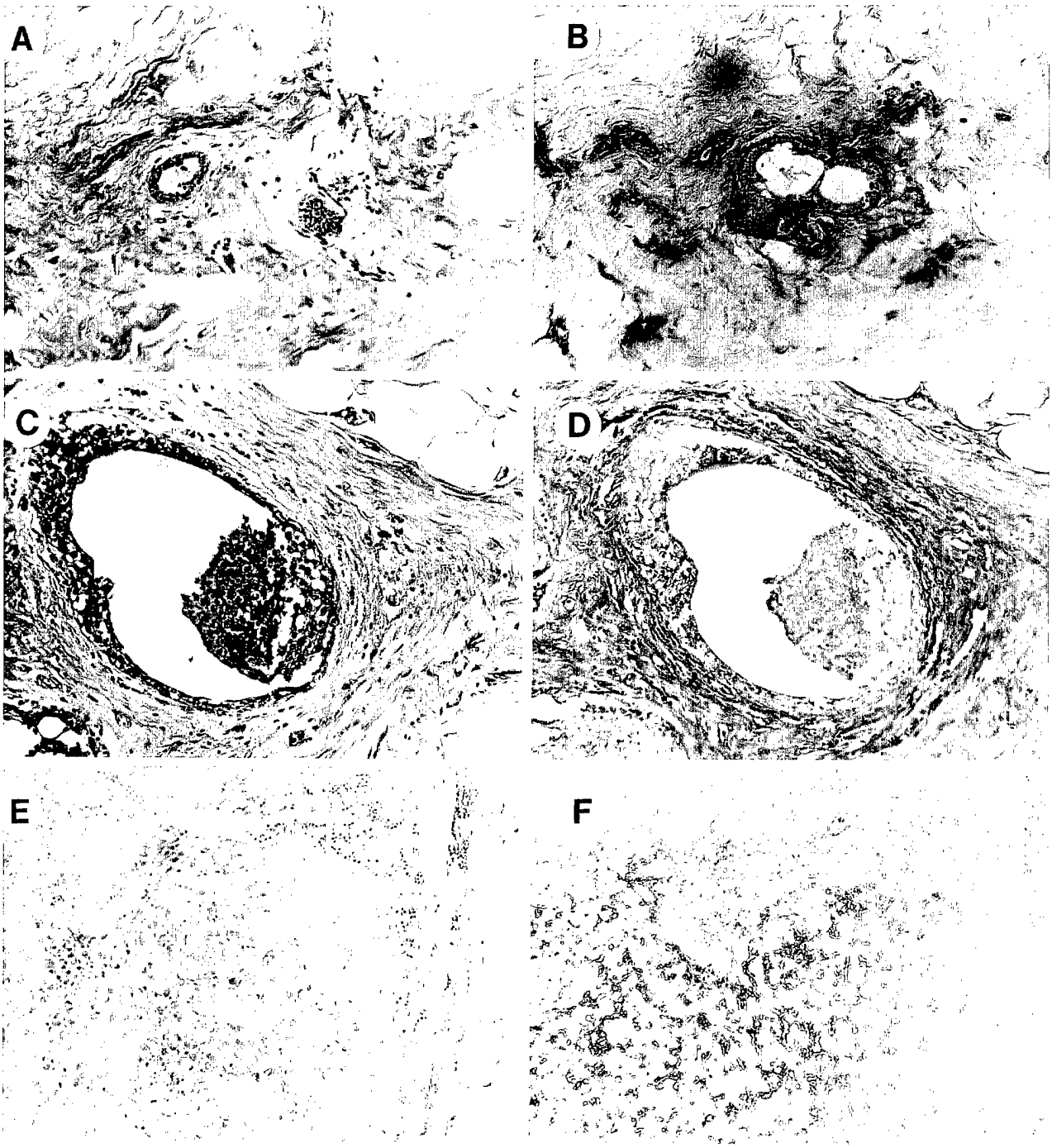

Fig. 10. The distribution of CD44 and HA in paraffin sections of tissue from a patient with breast cancer. The sections were processed as described in the legend to Fig. 9. (A) Normal breast epithelium shows variable amounts of CD44 (note: the staining in the stroma should be ignored). (B) HA is present in the stroma immediately surrounding the normal breast epithelium, but is absent from the stroma a short distance away. (C) This ductal carcinoma in situ expresses higher levels of CD44 than the normal tissue. (D) An adjacent section shows that HA is present in the surrounding stroma, but absent from the tumor mass. (E) A region of a lymph node shows that the metastasized tumor cells express only low levels of CD44. (F) An adjacent region shows that the tumor mass contains HA.

CONCLUSIONS:

Implication of Completed Research:

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) Large amounts of HA are associated with tumors that have metastasize to the lungs. This phenomenon may be useful for directing agents to the tumors.

Recommended Changes:

1) In Task 1, MCF-7 and ML-20 cells will be used instead of the ZR-751 cell line.
2) In Task 3, a mouse transfected with the polyomavirus middle T oncogene (45) will be used instead of the OncoMouse MM/v-Ha ras mammary carcinoma model (43). The frequency of tumor formation is greater in the former model system.
3) We propose to expand our investigation on the HA that is associated with the secondary tumors present in the lung tissue. We propose to determine whether it is derived from the tumor itself or from surrounding the normal lung tissue. In addition, we will determine if a similar phenomenon occurs in metastases to other tissues such as the liver.

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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.
BMS113 mAb	Monoclonan antibody to the standard form of human CD44 (appropriate for paraffin sections).
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.
pCMV5	Expression vector for mammalian cells carrying a cytomegalovirus promoter.
pCMV5-CD44	Expression vector containing sequence for human CD44.
pRc/CMV	Expression Vector containing neomyosin resistance.
pRc/CMV-CD44	Expression Vector containing sequence of CD44.
pSV_2-neo	Plasmid vector which confers neomyosin (G-418 sulfate) resistance - used as a marker for selecting cells that have taken up plasmids (including the PCMV5-CD44)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
SV-3T3	Simian virus 40 transformed mouse 3T3 cells (Swiss mouse).