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CD44 and Hyaluronan Expression in Human Cutaneous Scar Fibroblasts

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Fibrotic disorders of skin and other organs are typically associated with an abnormal accumulation of extracellular matrix. This study focuses on a matrix constituent, hyaluronan—which is known to be altered in fibrotic disorders of skin—and on CD44, a cell adhesion molecule and putative receptor for hyaluronan. Tissue samples were obtained from biopsies of human normal skin, normal cutaneous scar, and hypertrophic cutaneous scar. After culturing, cells were studied by single- and double-labeling immunohistochemistry using the two anti-CD44 monoclonal antibodies, BU-52 and J173, and a biotinylated hyaluronan binding complex probe, b-HABR. Certain cultures were pretreated with* Streptomyces *hyaluronidase to assess the dependency of CD44 expression on the presence of endogenous hyaluronan. CD44 expression, both in the presence and the absence of exogenous hyaluronan, was quantitated by radioimmunobinding assay. Overall glycosaminoglycan synthesis and identification of hyaluronan were accomplished by precursor incorporation assays and by quantitative cellulose acetate electrophoresis. CD44 was found to be a normal human adult fibroblastic antigen whose expression is markedly increased for hypertrophic scar fibroblasts compared with normal skin fibroblasts. Although hyaluronan was found to be the predominant glycosaminoglycan constituent of the pericellular matrix for these fibroblasts, CD44 attachment to the cell surface is neither mediated by hyaluronan nor is the presence of hyaluronan a prerequisite for CD44 expression. Exogenous hyaluronan induced a decline in measurable CD44 expression for normal skin fibroblasts but not for hypertrophic scar fibroblasts. These observations are compatible with current understanding of the way cells manage the hyaluronan economy of the extracellular matrix and emphasize phenotypic heterogeneities between fibroblasts derived from normal versus scar tissues. (Am J Pathol 1993, 142: 1041–1049)

Fibrotic disorders of skin and other organs are typically associated with an abnormal accumulation of extracellular matrix.[1] Increasingly, it appears that cellular receptors for specific matrix components can be identified on the surface of connective tissue cells and may participate in the recognition, binding, internalization, and catabolism of certain matrix constituents.[2] Comparing cells derived from normal versus fibrotic tissues would seem a logical step in establishing whether the existence, properties, and function of such receptors are implicated in matrix accumulation disorders and whether such receptors serve as cellular markers for different phenotypic behaviors among fibroblastic substrains. In pursuing this line of reasoning, we have focused on a matrix constituent, hyaluronan, which is known to be altered in fibrotic disorders of skin[3] and on CD44, a cell adhesion molecule and putative receptor for hyaluronan.[4]

In this study, we establish the presence of CD44 in human cutaneous scar tissues and demonstrate increased levels in scar fibroblasts compared with normal skin fibroblasts. This is the first study to demonstrate expression of CD44 on human cutaneous scar fibroblasts and to explore the relationship of CD44 and hyaluronan in such cells. A marked, time-dependent decline in CD44 expression was associated with preincubation of normal skin fibroblasts with hyaluronan. This may reflect a hyaluronan-induced down-regulation of CD44 for normal skin fibroblasts

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but not for hypertrophic scar fibroblasts—possibly indicating the predominance of different fibroblastic substrains in skin versus scar.

Materials and Methods

Tissue Samples and Cell Culturing

Tissue samples were obtained as biopsies of normal human skin, normal cutaneous scar, and hypertrophic cutaneous scar. In all cases, tissues were removed surgically as a part of treatment procedures. The freshly biopsied tissues were washed several times with Hanks' buffer and then cut into small pieces. Portions were taken for standard histological examination (stained with hematoxylin and eosin), whereas others were frozen in isopentane and cryosectioned for immunohistochemical studies. Remaining tissues were used to initiate fibroblast cultures by the methods of either Diegelmann et al[5] or Botstein et al.[6] Cells were subsequently counted in a Coulter counter and inoculated at a density of 5×10^5 cells/75 cm^2 tissue flask (Corningware, Corning, NY) in Dulbecco's modified eagle medium supplemented with 25 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, penicillin (100 μg/ml), streptomycin (100 μg/ml, Sigma Chemical Company, St. Louis, MO) and 10% fetal bovine serum. Cultures were fed twice weekly with the same medium (i.e., Dulbecco's medium plus fetal bovine serum) and subcultivated using 0.05% trypsin + 0.53 mol/L ethylenediaminetetraacetic acid. Previous experiments in our laboratory and elsewhere confirm that fibroblasts are the only cell type present after subcultivation; nevertheless, cultures were routinely examined with anti-vimentin and anti-fibronectin antibodies that are positive controls for fibroblasts and assure a monocellular fibroblastic phenotype. Cells were utilized during fourth passage. Three normal skin, three normal scar, and three hypertrophic scar cell lines were used.

Immunohistochemistry

Cultured fibroblasts were plated in Lab-Tek chamber slides (Miles Scientific, Naperville, IL) coated with poly-D-lysine (Sigma) to prevent cell detachment and loss. Each slide contains 8 distinct culture chambers, allowing staining to be performed for the variously treated fibroblasts derived from normal and scar tissues. While still subconfluent, media were removed, slides were washed with Hanks' buffer, and the slides were stained using the various monoclonal antibodies (MAbs) and probes.

Monoclonal Antibodies and Probe

Two anti-CD44 MAbs were used: BU-52 (The Binding Site, San Diego, CA) and J173 ascites, (a gift of Dr. John Pesando, The Biomembrane Institute, Seattle, WA). Both are mouse anti-human antibodies of the IgG1 isotype, and both were used at a concentration of 1:100. Two positive control MAbs were routinely used: anti-vimentin (at a concentration of 1:40) and anti-fibronectin (at a concentration of 1:100). Both were of the IgG1 isotype (Sigma). As a negative control, mouse IgG was used: To localize hyaluronan, a biotinylated form of the hyaluronan binding complex (b-HABR) was used at a concentration of 2 μg/ml in 10% calf serum, 90% phosphate-buffered saline (PBS). This complex, derived from cartilage proteoglycan, consists of hyaluronan binding region protein (HABR) + link and was a gift of Dr. Charles Underhill (Georgetown University Medical Center, Washington, DC).[7] A MAb was also used for the detection of a non-CD44 hyaluronan binding protein (HABP) possessing properties of a putative hyaluronan receptor molecule. This antibody, designated MAb IVd4, was a gift of Dr. Brian Toole (Tufts University, Boston, MA)[8] and was of the IgM isotype.

Single Labeling

The avidin-biotin horseradish peroxidase complex procedure of Hsu et al,[9] was used with modifications for each antibody tested. The primary antibody was incubated at 4 C overnight in a humidifying dark box (Accurate Chemicals, Wesbury, NY). After washing with buffer for 10 minutes with shaking, sections were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), diluted 1:200 in PBS, 0.1% sodium azide for 1 hour at room temperature, and then washed in buffer. Avidin-biotin-horseradish peroxidase complex (Vectastain ABC Kit Standard, Vector Laboratories) was placed on the slides for 30 minutes at room temperature and then washed. Sites of antibody reactivity were localized with the chromogens 3,3-diaminobenzidine (Sigma) or 3-amino-9-ethylcarbazole (Dakopatts, Carpenteria, CA). Positivity appeared as dark brown staining in the case of the former or bright red staining in the case of AEC. The reaction was terminated by dipping the slides in water and then counterstaining with Mayer's hematoxylin.

Comparisons in positivity for the different antibodies were made semiquantitatively as described previously[10] in which labeled cells within each high-power field were quantified per mm^2 using an

ocular grid micrometer. The proportion of labeled cells was then graded as: 0, 1+ (up to 25% labeled), 2+ (25–50% labeled), 3+ (50–75% labeled), or 4+ (75–100% labeled).

Double Labeling

The goal of the double labeling procedure was to simultaneously disclose both CD44 and endogenous hyaluronan on the cell surface. Anti-CD44 MAb and b-HABR probe were used. An original sequential double immunenzymatic labeling method modified after Hancock et al[11] was performed, again using chamber slides. CD44 was first localized by a four-layer peroxidase-anti-peroxidase method, consisting of incubations with MAb BU-52 (anti-CD44) at a concentration of 1:100. After incubation for one hour, the bridging antibodies, rabbit anti-mouse IgG and swine anti-rabbit IgG, were each incubated for 30 minutes, followed by the rabbit peroxidase-anti-peroxidase incubated for 30 minutes. Each layer was followed with several washes. For the chromogenic substrate reaction, AEC was again used to give a red color. Cells were then labeled with b-HABR probe using a 2-layer mouse alkaline phosphatase antialkaline phosphatase technique. After incubating overnight at 4 C and washing, slides were incubated with avidin-biotin-complex-alkaline phosphatase following the manufacturer's directions (Vector Laboratories). The substrate reaction was achieved using the alkaline phosphatase kit III (Vector Laboratories) to give a blue color. The reaction was terminated by washing in buffer; no counterstain was used. Red indicated CD44, whereas blue indicated hyaluronan. Color-mixed cells (red and blue substrates) expressing both antigens (co-labeled with both primary antibody/probe) appeared dark violet. Sections were washed and mounted in a 1:1 (v/v) mixture of glycerol and PBS. Control slides were obtained by omitting the primary antibody/probe in either labeling to show a lack of cross reaction between the first and second antibody sequences.

Enzymatic Pretreatment

Cells were seeded in chamber slides and allowed to grow for 24 hours. Media were then changed and replaced with media that contained either 0 or 20 turbidity reducing unit TRU/ml *Streptomyces* hyaluronidase (Calbiochem-Behring Corp., La Jolla, CA). Cells were incubated for 3 hours at 37 C. The enzyme reaction was stopped and slides were prepared for immunohistochemistry as described above to detect expression of CD44 and hyaluronan in hyaluronidase-treated and hyaluronidase-untreated cultures.

Radioimmunobinding Assay

A Dot Microfold apparatus (V&P Scientific Inc., San Diego, CA) was used to measure the expression of CD44 and HABP in normal skin, normal scar, and hypertrophic scar cell lines and to determine the effect of hyaluronidase pretreatment on CD44 expression. Cells either treated or untreated with *Streptomyces* hyaluronidase, as described above, were fixed with 2% glutaraldehyde for 30 minutes then seeded at 5×10^4 cells/well in the manifold. The manifold has the configuration of a 96-well plate whose bottom surface consists of glass microfilter paper (Whatman, Clifton, NJ). Five replicates of each condition and each cell line were used. Each well was incubated with 100 µl of anti-CD44 (BU-52) at a postsaturation concentration of 1:10 for one hour. This concentration was based on previous titration assays at concentrations of 1:100, 1:50, and 1:25 for each of the different cell lines. A negative control, in this case mouse IgG antibody, was also used. Wells were then washed with PBS, and the secondary antibody, a ^{35}S-conjugated sheep anti-mouse Ig, was used at 0.1 µCi/well. After 30 minutes, the wells were washed several times to remove nonspecific binding. Well sites were cut out separately from the filter, and each was counted for immune reactivity by liquid scintillation analysis. In another set of experiments, MAb IVd4 at a concentration of 1:50 was incubated for one hour with the different cell lines (each having been pretreated with *Streptomyces* hyaluronidase), and expression was quantified as mentioned above using ^{35}S-conjugated mouse Ig. Results were expressed as disintegrations per minute (DPM) for each cellular condition and each antibody tested.

Effects of Extrinsic Hyaluronan on CD44 Expression

The effect of cold (nonradioactive) hyaluronan on CD44 expression by normal skin and hypertrophic scar fibroblasts was examined: normal skin fibroblasts were seeded at 5×10^4 into 24-well plates. After 24 hours, the cultures were incubated with hyaluronan at two different concentrations (2 nmol/L and 20 nmol/L) at 37 C for 0 to 60 minutes. The cells were then washed, fixed with 2% gluteraldehyde, and incubated with BU-52 at 1:20 for 2 hours

at room temperature. A mouse IgG was used as a negative control. Binding of CD44 was revealed by incubation with ^{35}S-conjugated sheep anti-mouse Ig.

Similarly, hypertrophic scar fibroblasts were seeded at 5 × 10^4 into 24-well plates. After 24 hours, cells were incubated with 20 nmol/L of cold hyaluronan at 37 C for 0 to 360 minutes. Cells were then washed, fixed with 2% gluteraldehyde, and incubated with BU-52 at 1:20 for 2 hours at room temperature. Mouse IgG was again used as a negative control. The cells were then washed and incubated with ^{125}I-conjugated goat anti-mouse Ig (ICN Biochem, Costa Mesa, CA) for 1 hour, washed again, solubilized with 2N NaOH, and radioactivity counted.

GAG Synthesis

In fourth passage, three fibroblast cell lines derived from human normal skin, normal scar, and hypertrophic scar were seeded into three 75-cm^2 flasks (5 × 10^4 cells/10 ml) and cultured. After 48 hours, media were removed and cells were washed with Hanks' buffer, then incubated with ^{3}H-glucosamine (221 mCi/mmol, New England Nuclear, Boston, MA) at a concentration of 5 μCi/ml in serumfree medium. After 48 hours in culture, cells were harvested.

Labeled GAG from medium, pericellular, and cellular fractions were isolated as described previously.[12] Radioactivity for each fraction was measured by liquid scintillation analysis, and the results were normalized. Individual GAG constituents were then quantitated by electrophoresis on cellulose acetate plates as described by Cappelletti et al,[13,14] as modified by Bronson et al.[15]

Results

CD44 Expression

Qualitatively, simple visual inspection and scoring revealed that CD44 is expressed in cutaneous fibroblast cultures (Figure 1A). Cells from both uninjured skin and from normal and hypertrophic scar tissues exhibited cell surface reactivity for CD44 as disclosed by the CD44 MAbs, BU-52 and J173. Both

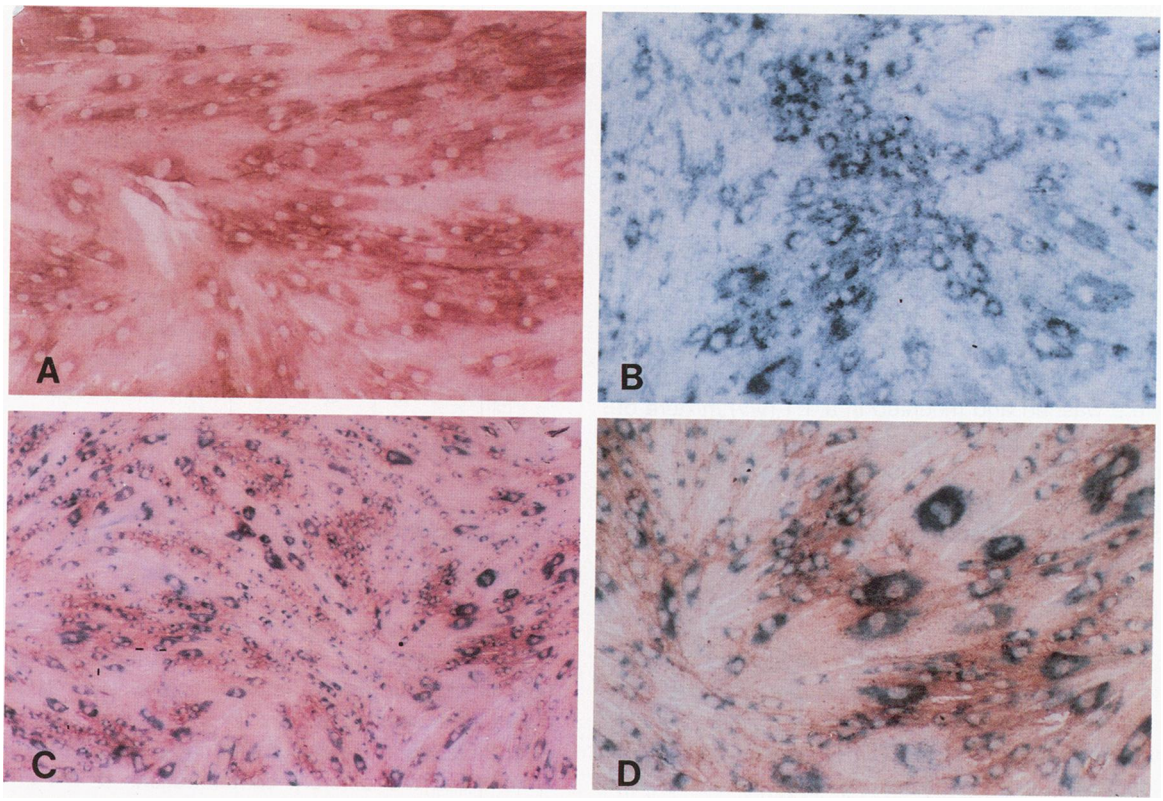

Figure 1. *Single immunocytochemical labeling of fibroblasts for CD44* **(A)** *using AEC as chromogen and for hyaluronate* **(B)** *using alkaline phosphatase blue substrate kit as chromogen. Note numerous CD44-positive cells as opposed to relatively few b-HABR-positive cells. Using double labeling techniques* **(C)**, *CD44+/HA+ cells seem to be restricted to a subpopulation of CD44+ fibroblasts.* **(D)**: *co-expression of both CD44 and hyaluronate on the same cells.* **(A, B)** *×200,* **(C)** *×100 and* **(D)** *×400.*

MAbs were diffusely expressed in 75% of the cultures with no difference in the pattern of distribution between them. When CD44 expression was quantitated by radioimmunobinding assay using ^{35}S-conjugated secondary antibody, a distinct difference in CD44 expression was seen. Hypertrophic scar fibroblasts showed the highest expression, whereas, normal (i.e., uninjured) skin fibroblasts showed the lowest ($P < 0.005$). CD44 expression for normal scar fell between these two extremes (Figure 2). The fibroblastic lines tested were not generically positive for hyaluronan-binding proteins (HABPs) inasmuch as they exhibited little reactivity to MAb IVd4 (developed from chicken embryo brain tissues), an anti-HABP antibody reactive for certain early embryonic tissues.

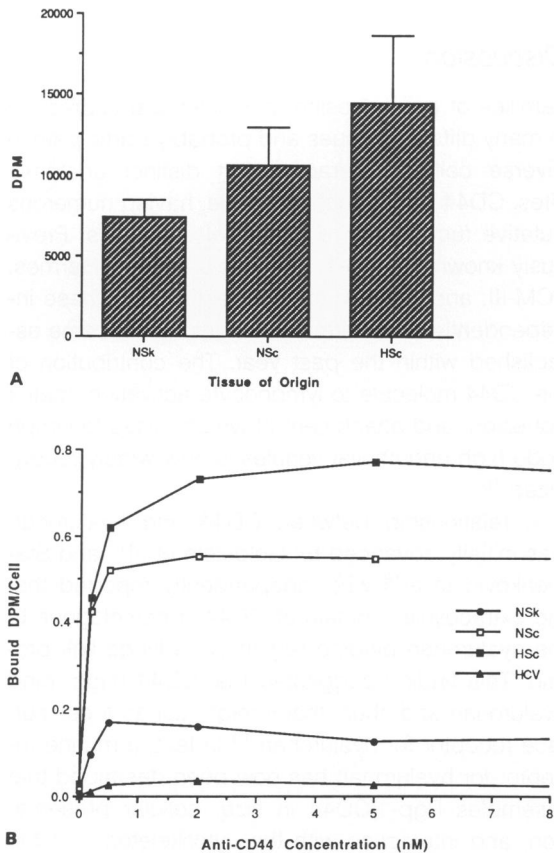

Figure 2. A: *Comparison of CD44 expression, for normal skin (NSk), normal scar (NSc), and hypertrophic scar (HSc) fibroblasts. Data are expressed as disintegration per minute (DPM) based on ^{35}S-conjugated mouse secondary antibody. A significant difference between normal skin and scar fibroblasts ($P < 0.005$) is observed using one-way analysis of variance. Values are expressed as mean ± S.E.* **B:** *Fibroblastic CD44 for cultures derived from normal skin (NSk), normal scar (NSc), and hypertrophic scar (HSc). A negative control cell line derived from invasive human bladder cell carcinoma (HCV) is also shown. Cells were seeded in complete media at a density of 3 × 10^4 cells/well in 24-well plates. Cultures were subsequently fixed with 2% glutaraldehyde for 30 minutes, washed and incubated with increasing concentrations (0 to 50 nmol/L) of mouse anti-CD44 IgG (MAb BU-52). After 1 hour of incubation, cultures were washed and then incubated for 1 hour with ^{35}S-conjugated sheep anti-mouse antibody (Ig). Each data point represents the average of values from triplicate wells. Bound DPM shown along the y-axis reflects the amount of cell surface CD44. Again, the fibroblasts derived from scar tissues are seen to be markedly higher in CD44 than are those from normal skin.*

Hyaluronan Expression

Using biotinylated b-HABR probe, hyaluronan was histochemically localized in cell cultures (Figure 1B). Some fibroblasts showed little or no reactivity, whereas others were strongly positive for hyaluronan—making it clear that hyaluronan expression is more variable than CD44 expression. This was true for all three categories of fibroblasts and was also evidenced by double-labeling immunocytochemistry (Figure 1D). Whereas 75% of the cells are CD44 positive (red color; Figure 1C), not all express hyaluronan. Only a subset of CD44-positive cells for each cell line were hyaluronan positive. The blue and red (violet) chromogen indicates those fibroblasts that co-express CD44 and hyaluronan.

Among all the GAGs produced by fibroblasts derived from normal skin, normal scar, and hypertrophic scar, hyaluronan was the most prominent (Figure 3). ^{3}H-glucosamine incorporated into nondialyzable (macromolecular) CPC-precipitable components (i.e., GAG) was detected in all three culture fractions (medium, pericellular, and cellular) for the nine cell lines tested. The majority (60%) of this incorporated label was secreted into the medium; the rest was associated with the pericellular matrix and intracellular components. For all three culture fractions, similar electrophoretic GAG profiles were obtained, and in all cases the predominant GAG electrophoresed in the position of hyaluronan. A secondary, lesser peak was seen in the position of dermatan sulfate. Hyaluronan is thus established as a prominent GAG found in the intra- and extracellular culture fractions of fibroblasts derived from both normal and hypertrophic scar tissues.

Relationship between CD44 and Hyaluronan

No direct relationship was seen between expression of CD44 and expression of hyaluronan. Nearly all the cells expressed CD44, whereas only a subset of fibroblasts expressed hyaluronan. Whereas clear differences were seen in CD44 expression between cells from the different tissues of origin (hypertrophic scar > normal scar > normal skin), no comparable relationship was seen for hyaluronan expression.

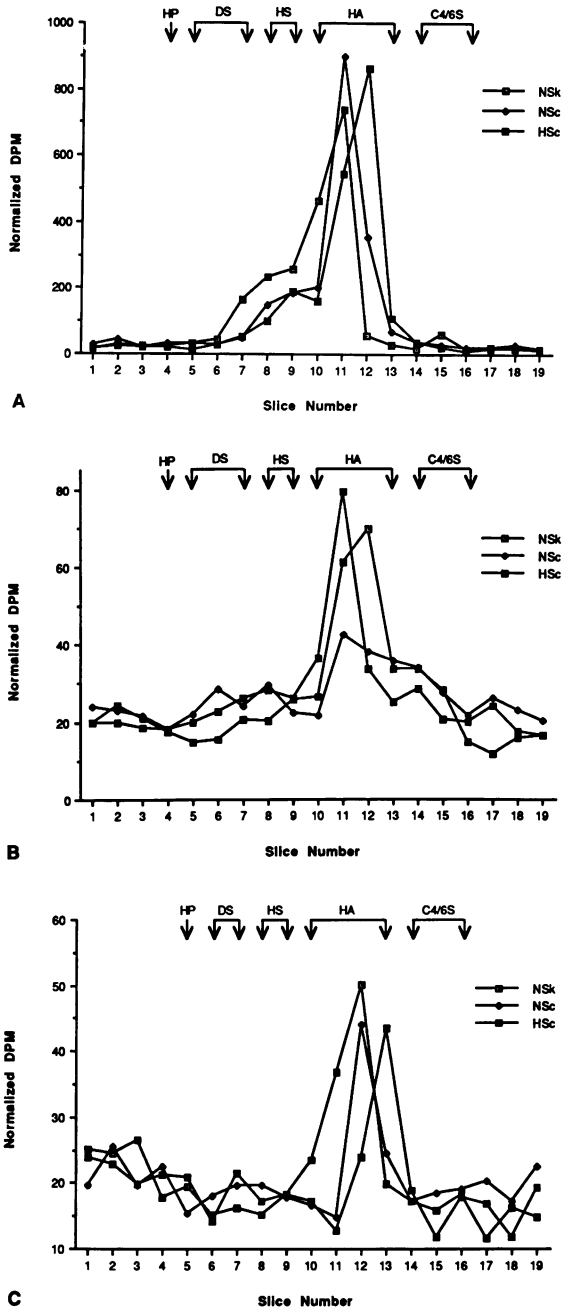

Figure 3. *Quantitative cellulose acetate electrophoretic profile of medium fraction* (A), *pericellular fraction* (B), *and cellular fraction* (C) *of subconfluent cells from human normal skin, normal scar, and hypertrophic scar, labeled for 48 hours with ^{3}H-glucosamine. The migration positions of known GAG standards are shown: HP = heparin, DS = dermatan sulfate, HS = heparan sulfate, HA = hyaluronan, C4/6S = chondroitin-4-sulfate and chondroitin-6-sulfate. Hyaluronan is shown to be the predominant GAG present in the medium, pericellular, and cellular fractions for all the cell lines.*

Streptomyces hyaluronidase pretreatment of cells did not abolish their CD44 reactivity to MAb BU-52 but did abolish staining for hyaluronan by b-HABR probe (Figure 4). Quantitatively, there was no significant difference in CD44 expression between cells treated with hyaluronidase and those untreated with hyaluronidase ($P > 0.1$) (Figure 5).

A relationship between CD44 expression and exposure of cultures to exogenous hyaluronan was observed (Figure 6). When normal skin fibroblasts were incubated with nonradioactive (cold) hyaluronan at two different concentrations (2 nmol/L and 20 nmol/L), a marked, time-dependent decline (down-regulation) of CD44 expression occurred. Hypertrophic scar fibroblasts cultivated under the same conditions did not exhibit a similar decline (Figure 7).

Discussion

Families of cell adhesion molecules are expressed in many different tissues and probably participate in diverse cellular interactions at distinct anatomic sites. CD44 is one such molecule, having numerous putative functions in a variety of cell types. Previously known as Pgp-1, In(Lu)-related p80, Hermes, ECM-III, and H-CAM, the co-identity of all these independently discovered molecules has become established within the past year. The contribution of the CD44 molecule to lymphocyte activation, matrix adhesion, and attachment of lymphocytes to lymph node high endothelial venules is now widely recognized.[16]

A relationship between CD44 and hyaluronan was initially advanced by Goldstein et al[17] and Stamenkovic et al[18] who independently reported that the extracellular domain of CD44 is homologous to the hyaluronan binding region of cartilage link protein. This finding suggested that CD44 might bind hyaluronan and, thus, that it might act as a cell surface receptor for hyaluronan.[19] In fact, a murine receptor for hyaluronan has now been described that resembles Pgp-1/CD44 in size, cellular presentation, and interaction with the cytoskeleton.[20,21] Miyake et al[20] and Aruffo et al[21] both confirmed a binding interaction between hyaluronan and CD44 and established CD44 as the principal cell surface receptor for hyaluronan in lymph node high endothelial cells. Aruffo et al[21] established hyaluronan as a ligand for CD44 based on the abolition of reactivity of hyaluronidase-treated lymph node tissue to a soluble CD44 receptor immunoglobulin fusion protein used as a probe for hyaluronan.

Such interactions between CD44 and hyaluronan have evoked special interest in view of hyaluronan's role in various biological processes, including cell-to-cell adhesion, cell migration during morphogenesis, and modulating the behavior of endothelial and

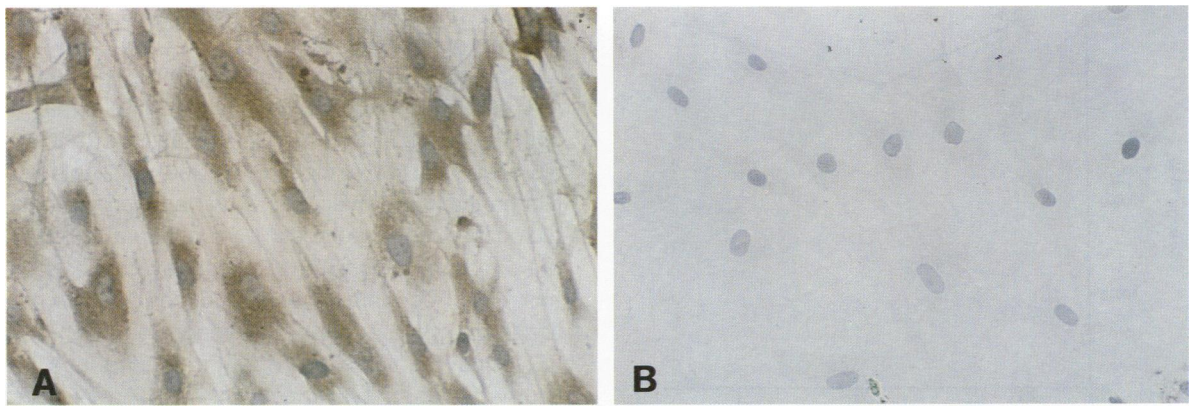

Figure 4. *Striking loss of (b-HABR) reactivity was observed when fibroblasts were treated with Streptomyces hyaluronidase. Immunohistochemical technique using 3,3-diaminobenzidine as chromogen was applied giving a dark brown coloration of the positive cells* **(A)**; *note complete loss of hyaluronate expression* **(B)** *after hyaluronidase treatment.* **(A, B)** ×*200.*

inflammatory cells,[22] particularly in the hyaluronan-enriched extracellular environment of early tissue repair and remodeling.[23] The present study examined the relationship between hyaluronan and CD44 in human adult skin and scar fibroblasts. It is the first investigation to report CD44 as a normal human adult fibroblastic antigen in culture and to assess human fibrotic tissues for the presence of CD44. Further, hyaluronan, as a known ligand for CD44, was shown to be the predominant GAG constituent of the pericellular matrix of fibroblasts derived from normal human and hypertrophic scar tissues. This is an important distinction between human and animal fibroblasts inasmuch as heparan sulfate predominates in the latter.[24]

Our previous work has indicated that hyaluronan binding to the surface of cutaneous scar fibroblasts seems to be a receptor-mediated phenomenon and is probably a prerequisite for hyaluronan endocytosis and degradation.[25] The accumulation of hyaluronan within the extracellular matrix depends on biosynthesis, transport, secretion, and degradation—all processes that may differ between fibroblasts from normal versus hypertrophic scar (or other fibrotic tissues). The presence and properties of hyaluronan receptors on such cells could be important in understanding mechanisms of hyaluronan binding to cell surfaces, subsequent internalization,

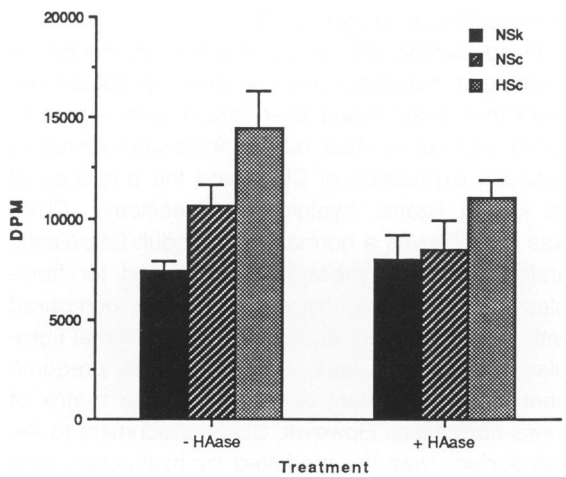

Figure 5. *CD44 expression in fibroblasts derived from normal skin (NSk), normal scar (NSc), and hypertrophic scar (HSc) tissues, untreated and treated with Streptomyces hyaluronidase (mean ± SE). Data are expressed as DPM of ^{35}S-conjugated mouse secondary antibody. These results show no significant reduction in the expression of CD44 in the different cell lines when treated with the hyaluronidase enzyme ($P > 0.1$) (t-test for paired samples).*

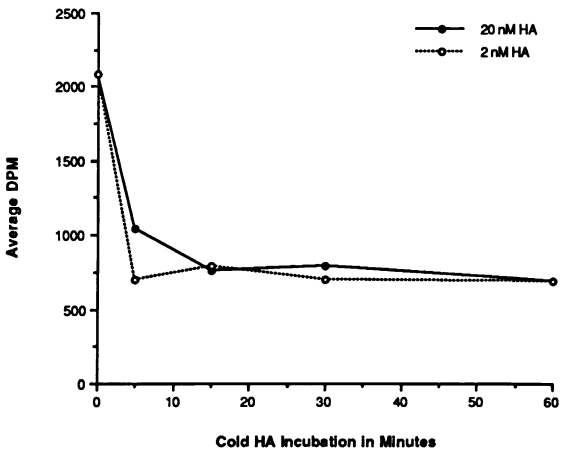

Figure 6. *Effect of incubation with nonradioactive (cold) hyaluronan on expression of CD44 by normal skin fibroblasts. Fibroblasts (5 × 10^4) were seeded into 24-well plates. After 24 hours, the cultures were incubated with hyaluronan at two different concentrations (2 nmol/L and 20 nmol/L) at 37 C for the periods shown (in minutes) along the x-axis. The cells were then incubated with BU-52 (anti-CD44) (1:20) for one hour. Binding of BU-52 was revealed by incubation with ^{35}S-conjugated sheep anti-mouse Ig and is expressed in DPM. A decline in CD44 is seen as the duration of incubation with hyaluronan increases.*

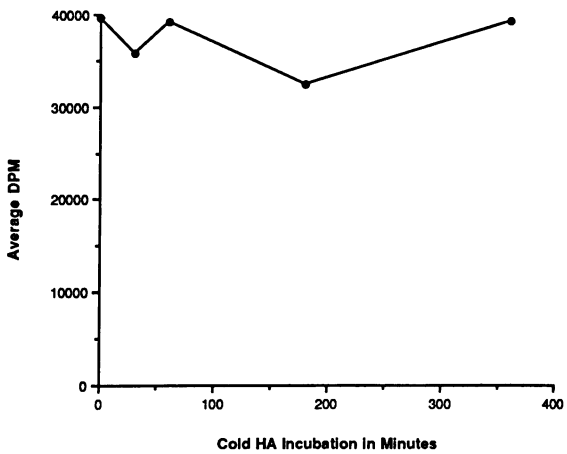

Figure 7. *CD44 expression for fibroblasts derived from hypertrophic scar as a function of duration of incubation with nonradioactive hyaluronan at a concentration of 10 nmol/L. Binding of BU-52 (anti-CD44) was revealed with ^{125}I-conjugated goat anti-mouse Ig. Binding is expressed in DPM along the y-axis; incubation of hyaluronan in minutes is expressed along the x-axis. For hypertrophic scar, no decline is seen as duration of incubation with hyaluronan increases.*

and degradation. In fact, Culty et al recently confirmed that CD44 participates in the uptake and degradation of hyaluronan by transformed fibroblasts (SV-3T3 cells) and alveolar macrophages.[26]

In the present study, *Streptomyces* hyaluronidase did not abolish CD44 reactivity but did abolish staining for hyaluronan with b-HABR probe. This result establishes that CD44 attachment to the cell surface is not mediated by hyaluronan and supports the concept of CD44 as an integral component of the cell membrane. If CD44 were simply an accretion to the hyaluronan-enriched pericellular matrix (which, in turn, is bound to the cell surface by some other means), then hyaluronidase treatment would have abolished both hyaluronan and CD44 expression. Thus, the CD44 molecule appears to be both distinct from hyaluronan and not dependent on the presence of hyaluronan for its expression.

We have demonstrated that normal skin fibroblasts express significantly less CD44 than do hypertrophic scar fibroblasts and that adding exogenous hyaluronan to cultures of normal skin fibroblasts causes a further decline in CD44 expression. A similar effect was not seen for hypertrophic scar cells. This prompt down-regulation of CD44 by normal skin fibroblasts but not by hypertrophic scar fibroblasts in response to external hyaluronan may explain why hypertrophic scar fibroblasts express higher levels of CD44 in the first place (Figure 2). Further, it supports the view that fibroblasts derived from normal skin and hypertrophic scar tissues belong to different fibroblastic substrains exhibiting different phenotypic behaviors.

In principle, a decline in CD44 expression by normal skin fibroblasts could have several explanations: 1) the anti-CD44 monoclonal antibody and hyaluronan may be competing for the same binding site on the CD44 molecule; 2) the antibody and hyaluronan may be competing for different epitopes, but exposure to hyaluronan effectively conceals the antibody's recognition site or induces a conformational change that alters its recognition site; or 3) CD44 may participate in both the binding and the internalization of hyaluronan, with binding involving the internalization of the entire receptor-ligand complex as has been shown in both endothelial and in 3T3 fibroblastic systems.[26,27] Among these three alternatives, the first and second have been excluded because incubating normal skin fibroblasts with increasing concentrations of nonradioactive hyaluronan over the range of 0 to 20 nmol/L did not cause a decrease in binding by the anti-CD44 antibody as would be expected if hyaluronan were either competing for the same recognition site as the anti-CD44 antibody or if it were concealing the recognition site (data not shown). These findings are compatible with the interpretation that normal skin fibroblasts internalize hyaluronan in the form of a receptor-ligand complex (i.e., CD44-hyaluronan), leading to less cell surface expression of CD44 when these cells are exposed to extrinsic hyaluronan. In contrast, hypertrophic scar fibroblasts do not down-regulate CD44 in response to hyaluronan—possibly because they do not internalize receptor-ligand or because they recycle receptors to the cell surface very quickly.

In conclusion, this study observed evidence for phenotypic heterogeneities between fibroblasts derived from normal and scar tissues with regard to CD44 expression. Also, relationships were identified between expression of CD44 and the presence of its known ligand, hyaluronan. Specifically, CD44 was identified as a normal human adult fibroblastic antigen whose expression is increased for fibroblasts derived from hypertrophic scar compared with those from normal skin. Unlike for animal fibroblasts, hyaluronan was shown to be the predominant GAG constituent of the pericellular matrix of these fibroblasts. However, CD44 attachment to the cell surface was not mediated by hyaluronan, and the presence of hyaluronan was not a prerequisite for CD44 expression. Moreover, exogenous hyaluronan induced a decline in measurable CD44 expression at the cell surface, which is not seen for hypertrophic scar fibroblasts. These observations are

consistent with current understanding of the way cells manage the hyaluronan economy of the extracellular matrix in other systems.

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