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Genetic Modulation of CD44 Expression by Intragraft Fibroblasts

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This study investigated the genetic composition and the functional implication of CD44 species expressed by intragraft fibroblasts. An LEW-to-F344 heart transplant model of chronic rejection was used. Intragraft fibroblasts recovered from the chronically rejecting allografts displayed a 4.5-fold increase in expression of CD44 mRNA when compared with that of the fibroblasts isolated from non-rejecting heart allografts ($P < 0.01$). The intragraft fibroblasts preferentially expressed CD44 variant isoforms containing v1 exon transcript. Automated nucleotide sequence analysis revealed that the majority (90.12%) of the CD44 v1 isoforms expressed by the rejecting graft fibroblasts were encoded by a mutated CD44 mRNA, which contained two point mutations and a codon deletion in the v1 coding region. Histochemistry demonstrated a massive deposition of extracellular HA in the rejecting heart allografts. Hyaluronic acid (HA) was able to promote *in vitro* fibroblast adhesion, migration in a CD44-dependent manner, and survival in a serum-free culture condition. The study concludes that up-regulation of CD44 v1 isoforms expressed by the intragraft fibroblasts is associated with an increase in the deposition of extracellular HA, the principal ligand for CD44, in the allografts, suggesting that CD44–HA interaction plays an important role in regulating fibroblast recruitment and growth in allografts developing chronic rejection.

Key words: CD44, gene expression, HA, intragraft fibroblast, rat.

Abbreviations: ECM, extracellular matrix; HA, hyaluronic acid; NG, non-rejecting graft; NH, native heart; RG, rejecting graft.

Clusters of differentiation antigen (CD) 44 is a transmembrane glycoprotein primarily involved in cell–cell interactions, cell adhesion and migration (*1–6*). It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens and matrix metalloproteinases (*7–10*). CD44 proteins are highly polymorphic. They are encoded by a single gene containing 20 protein-coding segments, including a group of standard and a group of variant exons (*10–12*). The standard exons are composed of exons 1–5 and 16–20, and expressed together on almost all cell types as the standard isoform (CD44s). The other group consists of 10 variant exons (exons 6–15) which can be alternatively spliced and included within the standard exons at an insertion site between exons 5 and 16. CD44 proteins containing the variant exons are designated as CD44v (*13*, *14*). Further diversity of CD44 proteins is generated by post-translational modifications, including glycosylation, phosphorylation or sulphation (*10*, *15*). Alterations in the induction and expression of CD44v isoforms have been associated with abnormal cell adhesion, growth and migration (*4*, *16*).

Fibroblasts are CD44-expressing cells of mesenchymal origin which widely distribute in different tissues and organs throughout the body (*3*, *15*, *17–19*). A prominent function of fibroblasts is to repair tissue following injury. During wound healing, fibroblasts are recruited into the wound site where they proliferate and differentiate into scar-forming myofibroblasts (*20*, *21*). The activities of the wound fibroblasts are regulated by a complex network of cytokines, adhesion molecules, metalloproteinases and the surrounding extracellular matrix (ECM) (*22*). Inactivated fibroblasts express basal levels of cell surface CD44, while the expression of CD44 by fibroblasts can be significantly up-regulated by cytokines (*23*, *24*). An increase in CD44 expression by fibroblastic cells was linked to an increase in cell adhesion on and migration through HA (*24*). Excessive fibroblast proliferation leads to fibrogenic disorders such as hypertrophic scar, liver cirrhosis and allograft fibrosis (*23*, *25*, *26*).

Graft fibrosis is a grave complication in long-term surviving allografts (*25*, *27–29*). The pathogenesis of allograft fibrosis is poorly understood and no effective regimen is preventative. We have recently conducted a series of investigations to characterize intragraft fibroblasts in order to understand their roles in chronic rejection. Data derived from studies using a LEW-to-F344 rat cardiac allograft model demonstrated that the majority of fibroblasts proliferating in the allografts are of host origin (*29*). Fibroblast progenitors originating from the mesenchymal compartment in the bone marrow travel via blood circulation and migrate into the allografts (*28*).

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In vitro studies further showed that migration of bone marrow stromal cells, which contain fibroblast progenitors relies on CD44–HA interactions (*24*). Based on these observations, we hypothesized that CD44–HA interaction is critically involved in the recruitment and growth of intragraft fibroblasts.

To examine the CD44 expression by intragraft fibroblasts, we isolated cardiac fibroblasts from heart allografts of LEW (donor) rats, which underwent chronic rejection by F344 recipients. CD44-specific cDNA libraries were constructed from the mRNA extracted from the isolated cardiac fibroblasts. Nucleotide sequence analysis of the cDNA libraries was performed to illustrate the genetic organization of the CD44 variant isoforms. In addition, the effects of CD44–HA interaction on the adhesion, mobility and proliferation of the intragraft fibroblasts were examined *in vitro*. The results derived from these studies demonstrate that expression of CD44 by intragraft fibroblasts was significantly up-regulated and the majority of CD44 variant isoforms contained exon v1 coding region. Up-regulated CD44 expression by the intragraft fibroblasts was coupled with an increase in deposition of ECM HA, the principal ligand for CD44 in the rejecting allografts.

MATERIALS AND METHODS

Animal Model—A cardiac transplant model of chronic rejection using Fischer 344 ($RT1^{lv1}$) rat as the recipient of a heart graft from LEW ($RT1^{l}$) rat was employed. Adult male or female animals weighing 200–250 g were purchased from Harland Sprague-Dawley (Indianapolis, IN, USA; www.harlan.com), housed in the rodent facility at Cedars-Sinai Medical Center, which is accredited by AAALAC International. All the animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Institutes of Health (NIH Publication No. 86-23, 1996 revision).

Microsurgical procedure for heterotopic heart transplantation was performed as described before (*30*). Heart allografts were evaluated daily by abdominal palpation.

Rejecting grafts (RG) were harvested at day 30 and non-rejecting grafts (NG) were harvested at day 5 post-transplantation, respectively. Native hearts (NH) were harvested alone with allografts and served as control.

Isolation of Cardiac Fibroblasts—Cardiac graft tissue was digested with a collagenase solution containing αMEM+ $CaCl_2$, 1mg/ml collagenases, 25 μg/ml DNase (1 ml/20 mg of tissue). The cells were re-suspended in 2% FBS–PBS. Macrophages were deleted from the single cell suspension by a procedure using an Easysep positive selective cocktail (anti-CD11b mAb at 100 μl/ml cells) according to the manual provided by the manufactory (StemCell Technologies, www.stemcelltechnologies.com). The cell suspension in which macrophages were deleted was then cultured in DMEM supplemented with 10% FBS for overnight. Culture medium and unattached cells were then removed by repeated washes. This procedure generates a population of dish adhering fibroblasts with >97% purity. The resulting cells exhibited typical fibroblast morphology, and were positive for αSmooth Muscle Actin (αSMA) (>85% with TGF-β stimulation) and vimentin (>97%) detected by immunocytochemistry.

Flow Cytometry—Intragraft fibroblasts were recovered from primary cultures and examined for CD44 expression in a flow cytometric antibody binding assay. The cells were stained with an FITC-conjugated anti-CD44 antibody (OX50, Chemicon, Temecula, CA, USA; www.chemicom.com) or an isotype match control (mouse IgG1-FITC) (Chemicon). Flow cytometric analysis of cell surface CD44 was performed using a FACSCalibur analyzer (Beckon Dickson Immunocytometry Systems, San Jose, CA, USA).

Quantitative Real-time PCR—Total mRNA from cells was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA, USA; www1.qiagen.com). Reverse transcription PCR was performed using 500 ng of total RNA samples with oligo dT primers (Roche, Indianapolis, IN, USA; www.roche.com). Quantitative amplification of the cDNA templates was carried out using a Quantitect SYBR Green PCR kit (Qiagen; webmaster@qiagen.com) in a TaqMan ABI PRISM 7300 real-time PCR system (Applied Biosystems, Foster city, CA, USA). The primer pairs used in this study for amplification of rat CD44 gene encoding the standard and variant isoforms (v1 to v10, respectively) and the conditions used in real-time PCR are as described previously (*24*). Primers for detection of hyaluronan synthase isoforms include: HAS1 (forward: 5′-TGGCCT TTGGGCTCTA TGG-3′; reverse: 5′-TCTGTGCCACTAG GTGCGC-3′), HAS2 (forward: 5′-TCGGAA CCACACTGTT TGGA-3′; reverse: 5′- AAGCAGCTGTGATTCCGAGG-3′) and HAS3 (forward: 5′-TGCCTACCAAGAGGACCCTG-3′; reverse: 5′-TGAGCTGAGC GAAGGCACTT-3′). Negative control was included in each run in which the template was replaced by an equal volume of water. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the loading control. The absolute levels of the mRNA were normalized with respect to GAPDH mRNA content. Samples per group were run in triplicate. To exclude non-specific amplification, the amplified samples were examined in electrophoreses on 1% agarose gel.

cDNA Libraries and Nucleotide Sequence Analysis—CD44 cDNA library was made from mRNA using a two-step RT–PCR procedure with CD44 forward primer E5 to generate single strain cDNA, and then with primer pairs E5 (forward) and E19 (reverse) to generate PCR products containing genes encoding rat CD44 isoforms. The CD44 E5/E19 PCR products were purified by Qiaquick gel purification kit (Qiagen). The purified PCR products were directly ligated into a pDrive cloning vector (Qiagen PCR cloning Kit), and followed by transforming EZ competent cell (Qiagen). Clones were screened for CD44 inserts using E5 and E19 PCR primers. Plasmids were purified by Maniple kit (Qiagen) and the plasmid DNA was sequenced using automatic DNA sequencing (Laragen.Inc, Los Angeles, CA, USA; laragen.com).

Detection of Recipient-derived Intragraft Fibroblasts—DNA was extracted from intragraft fibroblasts using a DNA extraction kit (Qiagen). The sex determining region at Y chromosome (*sry*) was detected in quantitative real-time PCR (qRT-PCR) with sry-specific primers described previously (*29*). Proportion of recipient-derived

fibroblasts (*sry*+) in total population of intragraft fibroblasts was determined by plotting the copy numbers of *sry*+ sample against a standard curve made from mixed populations of known numbers of male and female fibroblasts.

Cell Survival/Proliferation Assay—A simple cell culture-based proliferation assay has been modified for determining the cell survival with presence or absence of HA in the culture. Briefly, the test cells are exposed to a serum-free condition medium (FibroLife basal medium, Lifeline Cell Technology, Walkersville, MD, USA; www.lifelinecelltech.com) and subsequently cultured in a 96-well plate in which the wells were coated without or with HA-sodium salt (50 μg/well, Sigma Chemical, St Louis, MO, USA; www.sigma.com). At different time points the numbers of cells in culture are determined by using a CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA; www.promega.com). Comparison between cultures with or without addition of HA provides an indication of the cell survival in a nutrient-reduced condition.

Cell Adhesion Assay—Binding capacity of intragraft fibroblasts to HA gel was examined in a cell adhesion assay described previously (*24*). In the blocking experiments, cells were pretreated with an anti-CD44 antibody (10 μg/ml OX50, Chemicon) for 30 min at 37°C before plating. Isotype matched IgG1 (Chemicon) was used as a control.

Cell Migration Assay—Cell migration was examined in a Boyden chamber-based migration assay. Briefly, the insert membrane (Transwell®, 8 μm pore size, Corning In., NY, USA; www.corning.com) was coated with a HA solution (5 mg/ml), 100 μl/insert) and air-dried overnight. The test cells were re-suspended in a serum-free fibroblast culture medium supplemented with a LifeFactor supplement (Lifeline Cell Technology). An aliquot of 100 μl of cell suspension (5×10^5/well) was added to the upper chamber and the lower chamber was added with 200 μl of the same medium. After a 6 h incubation in the culture, the cells remained on the upper side of the membrane were wiped out by applying a cotton applicator. The migrating cells appeared in the lower chamber including cells on the other side of the membrane were treated with 40 μl of a combined MTS tetrazolium salt (MTS)/PMS solution (Promega Corporation). The cells in the lower chamber were incubated for 2 h at 37°C in a humidified, 5% CO_2 atmosphere. The culture medium was then transferred into wells of a 96-well plate (100 μl/well) and recorded the absorbance at 490 nm using an ELISA plate reader. The inhibitory effect of anti-CD44 antibody on the motility of the Intragraft Fibroblast (IF) was examined in an antibody blocking experiment in which the cells were pre-treated with an anti-CD44 antibody (10 μg/ml OX50, Chemicon) for 30 min at 37°C before plating into the migration chambers. Isotype matched IgG1 (Chemicon) was used as a control.

Metamorphic Analysis of Intragraft HA Deposition—HA deposition in heart grafts was demonstrated by incubation of the cryostat sections (6 μm in thickness) of the heart grafts with a biotin-conjugated HA binding protein (HABP, Seikagaku America, East Falmouth, MA, USA). Control slides were pre-incubated with hyaluronidase (50 U/ml) at 37°C overnight and followed by HABP staining. Colourization was performed using a staining procedure with a streptavidin-HRP kit (Zymed, CA, USA). An AEC (3-amino-9-ethylcarbazole) substrate for HRP was used as chromogen. In parallel, sections stained by the procedure with omission of bHABP served as a negative control. Ten randomly selected snapshot microphotographs (400× magnification) for each sample were obtained through a light microscope (Nikon E80i) equipped with a computerized image system. The micro-photographic images were analysed for integrated optical density of the HA staining using a Metamorphic program (Molecular Devices, Downingtown, PA, USA; www.moleculardevices.com).

Soluble HA Detection by ELISA—HA secreted by fibroblasts in conditioned medium was quantified using an HA ELISA kit (Echelon Biosciences Inc, Salt Lake City, UT, USA; www.echelon-inc.com). Briefly, intragraft fibroblasts were seeded in a 6-well plate and cultured for 48 h. Condition media (supernatant) were then collected for ELISA, while the remaining cells were trypsinized for cell count. The principle of HA measurement is based on competitive ELISA in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. The binding percentage for each sample is calculated using a formula: [A405(sample) – A405(Blank)]/[A405(Zero HA) – A405(Blank0)] × 100% = percentage of binding. The concentration of HA was determined by plotting each sample's binding percentage against HA standard curve as described by manufacturer's manual.

Statistics Analysis—Results of experimental data were reported as mean ± SD. Significance levels were determined by Mann–Whitney test using Medicalc Statistic software (MedCalc, Belgium; www.info@medcalc.be).

RESULTS

Sample Collections and Histological Examination—Nine heart allografts from female LEW rats to male F344 recipients were harvested at day 30 and five allografts harvested at day 5 post-transplantation. Five native hearts were collected from the allograft recipients and served as control. Histological examination demonstrated that all the day 30 allografts exhibited histological changes of chronic rejection, including interstitial mononuclear cell infiltration, fibroproliferation and development of vasculopathy. Non-rejecting allografts harvested at day 5 post-transplantation did not display histological changes of allograft rejection. Samples of native hearts exhibited normal cardiac histology.

High Levels of CD44 Expression by Intragraft Fibroblasts—As shown in Fig. 1, intragraft fibroblasts recovered from the chronically RGs ($n = 9$) displayed a 4.5-fold increase in the expression levels of CD44 mRNA when compared with that of the fibroblasts isolated from NGs ($n = 5$, $P < 0.01$), and a 3-fold increase over the cardiac fibroblasts from the NH ($n = 5$, $P < 0.001$). Consistently, results (Fig. 1C) of flow cytometry showed that fibroblasts recovered from the RG ($n = 4$) expressed significantly higher levels of cell surface CD44 (mean channel shift: 65.26 ± 7.03) than that observed at the fibroblasts from the NH ($n = 3$; mean channel shift: 10.46 + 2.46, $P < 0.001$) and the NG ($n = 3$; mean channel shift: 12.76 + 5.59; $P < 0.001$).

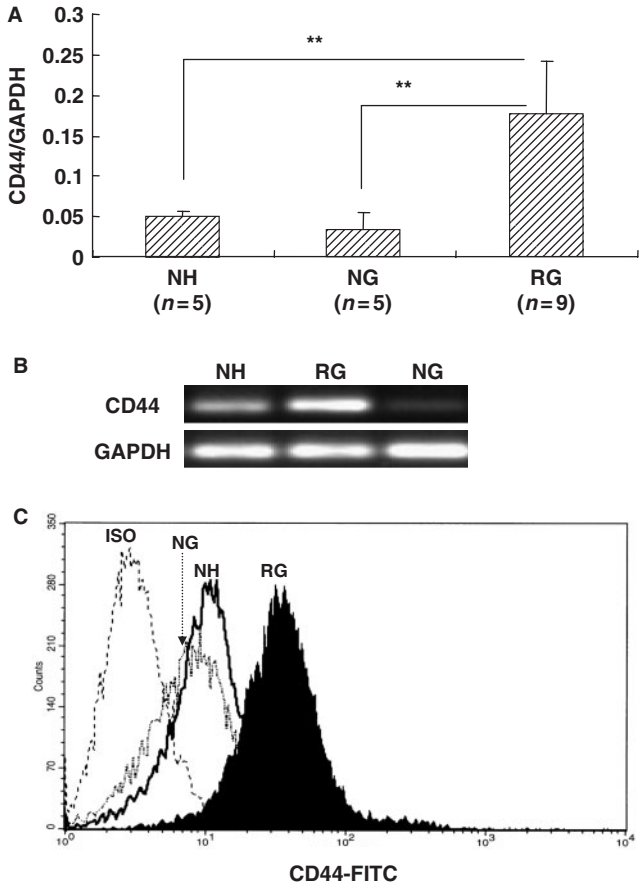

Fig. 1. **Intragraft fibroblasts isolated from the RGs expressed significantly higher levels of CD44, when compared with the fibroblasts from the NGs or the fibroblasts from NHs.** (A) CD44 E5 mRNA expression detected by qRT-PCR. Expression levels were normalized against GAPDH. (B) A representative PCR reaction product was visualized following agarose gel electrophoresis. (C) Flow cytometric detection of cell surface CD44 using an FITC-conjugated antibody OX50. Iso, isotype control; $^{**}P < 0.01$.

Differential Expression of CD44 Variant Isoforms by Intragraft Fibroblasts—The expression patterns of CD44 variant isoforms were studied using qRT-PCR (Fig. 2A). Approximately one-third of the CD44 proteins were expressed as variant isoforms. Significant differences in CD44v utilization were noted at isoforms containing exons v1 ($P < 0.001$) or v6 ($P < 0.01$) between the RG group and the NG group. The precursor frequency of individual v exons used by the CD44 v isoforms expressed by the intragraft fibroblasts from the rejecting grafts was estimated as v1 (42.0%), v2 (3.8%), v3 (7.0%), v4 (5.4%), v5 (5.9%), v6 (18.6%), v7 (4.7%), v8 (3.9%), v9 (5.3%) and v10 (3.3%). In comparison, the precursor frequency for the fibroblasts from the NGs was v1 (20.1%), v2 (1.3%), v3 (5.8%), v4 (22.9%), v5 (10.5%), v6 (13.9%), v7 (13.7%), v8 (6.8%), v9 (3.3%) and v10 (1.6%).

To further study the genetic composition of CD44 species expressed by intragraft fibroblasts, CD44-specific cDNA libraries were constructed from the cardiac fibroblasts isolated from three rejecting heart allografts. Nucleotide sequence analysis was performed on 103 clones randomly selected. As dispatched in Table 1, 64 clones (62.1%) contained sequences coding for CD44 standard isoform, while 39 clones (37.9%) coding for variant isoforms. Among the variant isoforms for which nucleotide sequences were determined, only one clone contained a combined v1 and v6. The rest of the CD44 v isoforms were encoded by a single variant exon along with the standard exons. Exon v1 was present at 84.6% of the CD44 v isoforms while v6 at 15.4%. It is worth noting that, 30 (90.1%) of a total 33 v1-containing isoforms sequenced exhibited point mutations at position 713 (A to G) and 774 (T to G), and a codon (AGC) deletion at position 775–777, which was located next to the second point mutation (Fig. 2B). Alignment of the amino acid sequence deduced from the mutated CD44 v1 with the wild-type protein sequence demonstrated replacements of a K (lysine) to R (arginine) at position 238, a S (serine) to E (glutamic acid) at position 249 and a deletion of a D (aspartic acid) at position 248 of the peptide (Fig. 2C).

Increase in HA Deposition in the Rejecting Cardiac Allografts—Presence of extracellular HA was demonstrated by staining of the cryostat sections of cardiac allografts with HA binding protein. As displayed in Fig. 3, native hearts and non-rejecting heart allografts exhibited scattered spots of interstitial HABP staining. In contrast, intensive staining of HABP was found in all the rejecting cardiac allografts examined. Staining of HABP was localized both in interstitial tissue and myocardium. Pre-digestion of the tissue sections with a hyaluronidase diminished HABP staining. The intensity of HABP staining of cardiac grafts was further quantitatively analysed using a metamorphic scanning program (Fig. 3D). The levels (as displayed as integrated density) of HABP binding to the RGs ($1.6 \pm 0.37 \times 10^9$; mean ± SD) were significantly higher than the levels of NGs ($0.19 \pm 0.003 \times 10^9$; $P < 0.001$) and NHs ($0.19 \pm 0.002 \times 10^9$; $P < 0.001$).

Up-regulation of HA Synthase Gene Expression and Increase in HA Secretion by RG Fibroblasts—Cardiac fibroblasts derived from RG, NG and NH were investigated for their contributory role to HA deposition in chronically rejecting heart allografts. The results (Fig. 4A) of quantitative PCR show that HAS2 is the major isoform involving in HA synthesis in cardiac fibroblasts. RG fibroblasts expressed significantly higher levels of mRNA coding for HAS1, HAS2 and HAS3 when compared with fibroblasts derived from NG ($P < 0.01$ for HAS1 and HAS2, respectively) and NH ($P < 0.01$ for HAS1, HAS2 and HAS3, respectively). Up-regulated HA synthase expression was coupled with an increased HA secretion by RG fibroblasts. As demonstrated by the results of ELISA (Fig. 4B), HA concentration was significantly increased in RG conditioned medium (262.3 ± 7.8 ng/10,000 cells) when compared with conditioned medium of NG (115.2 ± 15.1 ng) or NH (51.9 ± 6.6 ng, $P < 0.01$).

HA Sustained Cardiac Fibroblast Survival in Culture—Intragraft fibroblasts isolated from the rejecting heart allografts ($n = 6$) and NGs ($n = 3$) were cultured in a serum-free medium without refreshment for up to 8 days. Viable cells were determined in a MTS-based

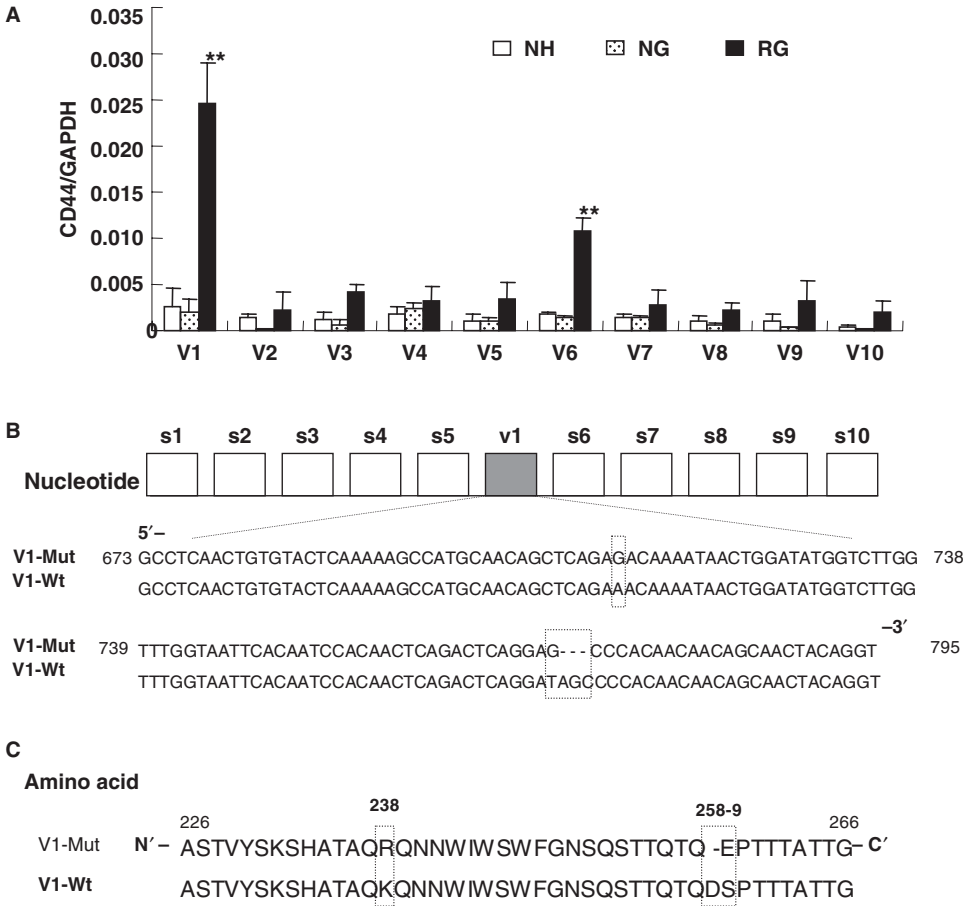

Fig. 2. **Preferential expression of CD44 variant isoforms containing exon v1 and/or v6 by the intragraft fibroblasts isolated from the rejecting cardiac grafts.** (A) Variant exon expression by intragraft fibroblasts detected by qRT-PCR. Note the levels of exon v1 and v6 expression by fibroblasts isolated from the rejecting allografts ($n = 9$) were 4.5-fold and 3-fold higher than that of the non-rejecting allografts ($n = 5$) or native heart fibroblasts ($n = 5$); $^{**}P < 0.01$. (B) Nucleotide sequence alignment of a mutated and a wild-type v1 coding region. The drawing shows the genetic organization of CD44 v1 isoform. Note the insertion site of v1 exon transcript locates between s5 and s6. (C) Expected amino acid sequence of the mutated CD44 v1 region. The mutations are indicated by a box. The nucleotide and amino acid sequence alignments were originally performed on a Nucleotide Blast program with the rat CD44 gene sequence U96138.

Table 1. **Isoform composition in 103 CD44 clones determined by automate nucleotide sequence analysis.**

Isoform	Clones number	Percentage	Mutation
CD44 standard	64	62.1	No
CD44v1	33	32.0	Yes
CD44v6	5	4.9	No
CD44v1,v6	1	0.97	No
Total	103	100	NA

proliferation assay. As demonstrated in Fig. 5, intragraft fibroblasts from the RGs in cultures with HA supplement exhibited a 3-fold increase in cell numbers at day 4 and were able to maintain the cell population size for up to 8 days. In comparison, the same intragraft fibroblasts in cultures without HA exhibited a lower growth curve after 3 days in the culture and started to decline in cell numbers at day 5. There were significant differences in cell numbers between the cells with and without HA supplement at days 5 through 8, indicating that HA could promote fibroblast survival. In addition, fibroblasts recovered from the NGs exhibited a lower proliferation curve when compared to the cells from the RGs.

HA Promoted Fibroblast Adhesion and Mobility—As shown in Fig. 6, the intragraft fibroblasts recovered from the rejecting grafts exhibited higher binding ability to HA gel when compared with that of the fibroblasts from the NGs ($P = 0.007$) or the NH fibroblasts ($P = 0.045$). The binding of the RG cells to HA was partially blocked by anti-CD44 antibody OX50 ($P = 0.027$). Intragraft fibroblasts isolated from the RG and NG also exhibited significant increases in mobility when the transwell filters were coated with HA ($P = 0.01$ and $P = 0.004$, respectively), suggesting their HA dependency. Furthermore, migration of RG, but not NG fibroblasts was significantly blocked by OX50 antibody ($P < 0.001$).

The Majority of Intragraft Fibroblasts were Recipient-derived—DNA samples extracted from the intragraft fibroblasts from cardiac allografts were examined for

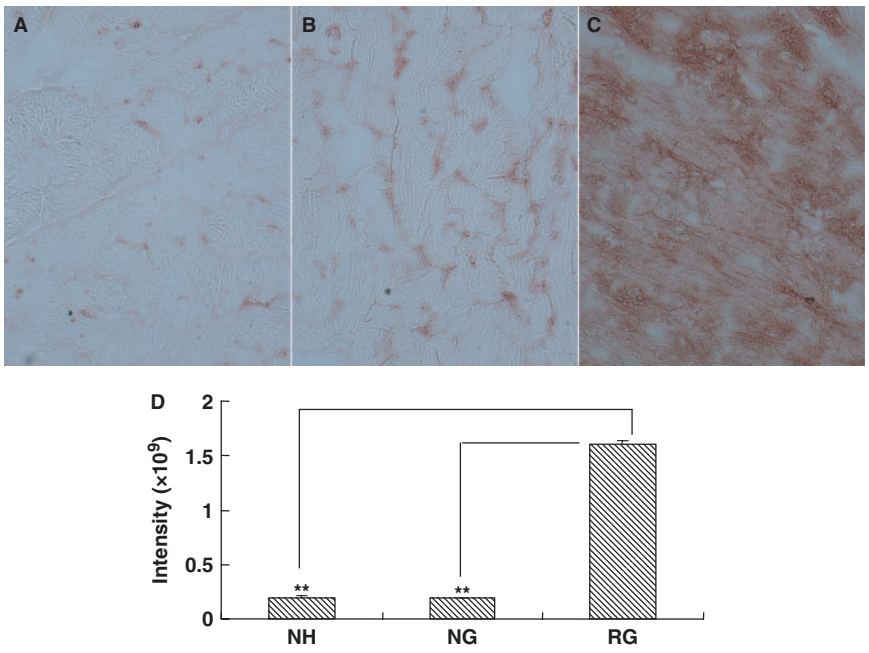

Fig. 3. **Increased HA deposition in heart allografts with chronic rejection detected by HABP staining.** (A) A NH and (B) a non-rejecting heart graft displayed minor interstitial HABP staining. (C) A rejecting heart allograft exhibited heavy interstitial HABP staining. All the micrographs are present at original magnification of 200×. (D) Metamorphic scanning analysis of HABP stained tissue sections. The data were derived from experiments on five rejecting allografts, five non-rejecting allografts and five NHs. Ten randomly selected micrographs at 400× magnification for each graft were analysed; $^{**}P < 0.01$.

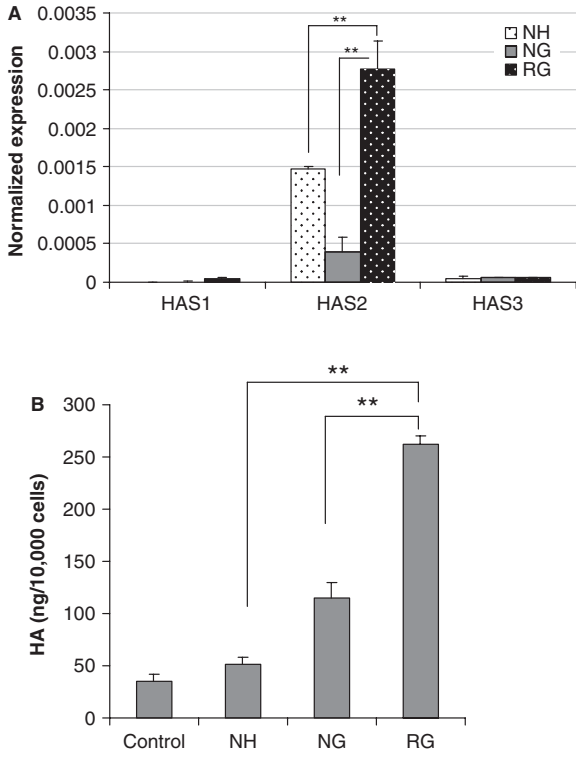

Fig. 4. **RG fibroblasts secreted high levels of HA.** (A) Up-regulated expression of HA synthase isoforms by RG fibroblasts detected by qRT-PCR. Note that HAS2 is the main HA synthase expressed by cardiac fibroblasts. (B) An increase in HA concentration in RG fibroblast conditioned media detected by competitive ELISA; $^{**}P < 0.01$.

the presence of *sry* gene in the Y chromosome by qRT-PCR. The *sry* gene was detected in all of the samples. The incidence of *sry*+ cells in total cell population was estimated by plotting the gene copy numbers obtained from allograft samples against a standard curve, which represents a series of known quantities of the *sry* gene. The results showed that male cells in the intragraft fibroblast populations accounted for $14.87 \pm 5\%$ in the NGs ($n = 3$), which were harvested at day 5 post-transplantation and $55.58 \pm 6.9\%$ in the RGs ($n = 9$), which were harvested at 30 days post-transplantation.

DISCUSSION

The current study demonstrated an up-regulated CD44 expression by intragraft fibroblasts isolated from chronically rejecting rat heart allografts. A 4.5-fold increase in the level of CD44 mRNA was found in the RG fibroblasts over the NG fibroblasts. The qRT-PCR data are further supported by the FACS results. A similar trend of increases in cell surface CD44 proteins was observed in rejecting allograft fibroblasts. Fibroblasts expressing CD44 have been previously reported during the wound healing process in which the cells were recruited into the wound site (*21*). An augmentation in cell surface CD44 expression by the intragraft fibroblasts from the RGs suggests an increase in CD44 signalling, which may lead to a wide spectrum of cellular activities, including cell–matrix interaction, migration, differentiation and proliferation (*15*, *31*). Thus, quantification of CD44 expression in transplant biopsies could have a diagnostic value for detection of chronic rejection and allograft fibrosis.

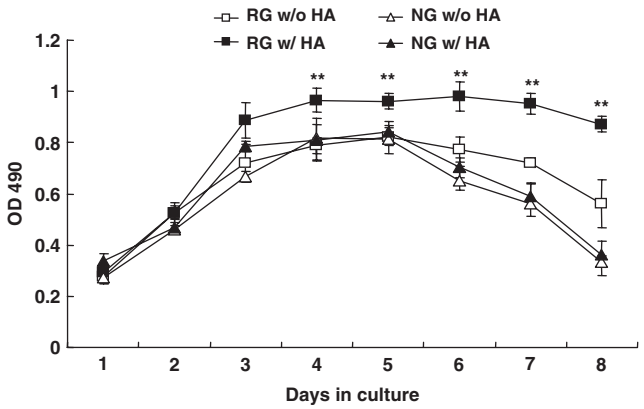

Fig. 5. **HA sustained the survival of intragraft fibroblasts in serum-free condition medium.** Note that fibroblasts isolated from the RGs in cultures with HA supplement (RG w/HA) tripled in cell numbers at day 4 and exhibited significant differences ($^{**}P < 0.01$) from the same cells in cultures without HA (RG w/o HA) from days 4 though 8. In comparison, fibroblasts isolated from the non-rejecting heart allografts displayed a lower growth curve and a poor response to HA addition.

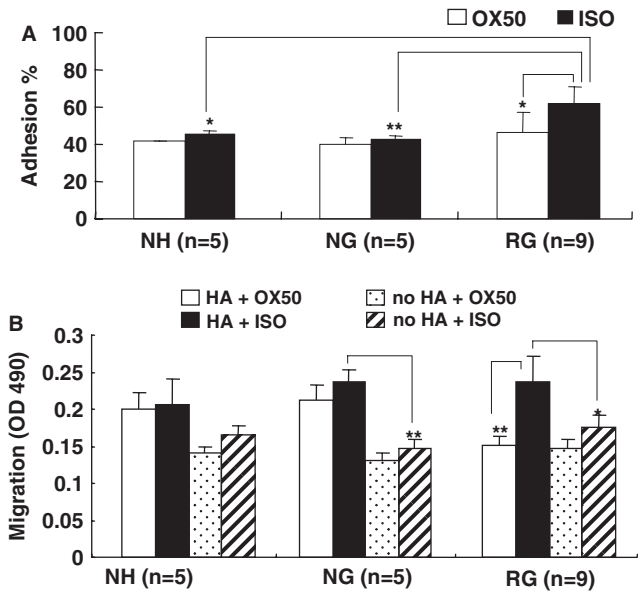

Fig. 6. **Intragraft fibroblasts derived from the rejecting heart allografts exhibited increases in the abilities of adhering to and migrate through HA gel.** (A) Adhesion assay, showing that the percentage of cells binding onto the plate was significantly higher in the rejecting graft group than the non-rejecting group. Anti-CD44 antibody (OX50) partially blocked the cell adhesion. (B) Migration assay. Note that HA coating promoted cell migration in both the RG and the NG groups, whereas OX50 treatment significantly reduced RG but not NG fibroblast migration. $^{*}P < 0.05$, $^{**}P < 0.01$.

The current qRT-PCR data demonstrated that the fibroblasts from the RG not only markedly increased the bulk of CD44 production but also exhibited a unique CD44v expressing profile, which is characterized by a preferential utilization of exons v1 and v6, in less extent, for coding the variant isoforms. Expression of the v1 exon by intragraft fibroblasts was 10-fold higher in the RGs than in the NGs. This profile of CD44v expression is obviously different from the CD44v profile we previously observed in a rat mesenchymal stem cell line Ap8c3, in which CD44 standard isoform is predominantly expressed (*24*). Induction and up-regulation of CD44v isoforms has been linked to changes in cell biobehaviours such as proliferation, differentiation, migration and tumour cell invasion (*32*). The CD44v expression profile observed in this study indicates a bias in the use of v1 exon by the RG fibroblasts. The biological implication of such biased expression of CD44 v1 isoform is not clear at this moment. Based on the observations that *sry+* recipient fibroblasts increased from 17% in the non-rejecting allografts to 55% in the rejecting allografts and a corresponding increase in CD44 v1 expression by the RG fibroblasts, it is possible that the migrating fibroblasts from the recipients were the cells expressing the CD44v1 isoforms.

The DNA sequencing data suggested new insights into the genetic structures of CD44 species expressed by the RG fibroblasts. First, the vast majority (97.4%, 38 out of 39 clones) of the variant isoforms examined contained an insert of mRNA sequence from a single variant exon. Only one clone contained two variant exons (a v1 and a v6) and no isoforms contained three or more v exons (Table 1). The data suggest that the intragraft fibroblasts express relatively simple CD44v isoforms. In contrary, CD44v isoforms encoded by several v exons have been reported previously (*4*, *33*). Ectopically expressed CD44 v4-7 variant containing v exons 4, 5, 6 and 7 was found to confer metastatic behaviour to normal cells (*34*). Expression of CD44 variant isoforms containing v6, v8-10 by hepatic fibroblasts (stellate cells) was associated with the development of cholestatic cirrhosis in rats (*19*).

Second, CD44v isoforms containing v1 exon were predominantly expressed by the rejecting allograft fibroblasts. Of 39 CD44v clones, 33 (84.6%) of them contained exon v1. CD44 proteins containing certain variant exon products are thought to be associated with specific cellular activities. For example, a CD44v isoform containing v6 sequence is strictly required for c-met activation by HGF in rat and human cells (*35*). Expression of CD44 isoforms containing v5 correlates with enhanced malignancy and invasiveness of some tumours (*36*). Certain CD44v3-containing isoforms are involved in the binding of heparin binding growth factors, such as basic fibroblast growth factor and vascular endothelial growth factor (*33*). The functional aspect of CD44v1 isoform expressed by the intragraft fibroblasts is currently unknown. We recently conducted a siRNA study to explore a possible role of CD44v1 isoforms in fibroblast proliferation. Our results (see Supplementary Material) demonstrated that while siRNA CD44e5, which interferes all the isoforms of CD44 had suppressive effect on HA-dependent growth of RG fibroblasts, SiRNACD44v1, which is designed to target v1 containing CD44 variant isoforms was ineffective. The data suggest that the induction of v1 exon sequence into the CD44 molecule could not alter the growth behaviour of RG fibroblasts.

Finally, mutations were identified in 30 (91%) out of 33 clones containing the CD44v1 mRNA sequences. The mutations included a codon triplet (AGC, Aspartic acid) deletion and two point mutations, which resulted

in amino acid alternations. All the mutations appeared at the region encoded by variant exon 1. The mutations are validated by searching the gene bank with the Nucleotide Blast program. Information regarding CD44 mutation is limited in the literature. One report showed that a mutation in a CD44 variant of inflammatory cells enhances the mitogenic interaction of FGF with its receptor (*16*). The mutated CD44 variant encoded an extra trinucleotide (CAG) transcribed from intronic sequences flanking a variant exon (*16*). The biological emphasis of the mutations observed in the current study is not clear. Transduction of CD44 null fibroblasts to ectopically express the mutated v1 isoform and subsequent functional assays will further aid in understanding the functions of this mutated V1 variant. On the other hand, human CD44 gene variant exon 1 is constitutively silenced due to an insertion of a stop codon at the 17th amino acid (*12*). Thus, the variant exon 1 is seemingly under constant evolution pressure. It is well established that polymorphic CD44 proteins can be generated by either alternative pre-mRNA slicing or post-translational modifications such as glycosylation, phosphorylation or sulphation (*2*, *10*, *11*). The current data suggest that somatic mutation in the variant exons may also serve as an additional mechanism to generate diversified CD44 species.

An increase in CD44 expression by the intragraft fibroblasts was associated with an augmentation in the cellular functions. Increased activities in HA adhesion and migration through HA-coated transwells were observed in the RG fibroblasts. Anti-CD44 antibody OX50 was able to partially block the adhesion and migration of the RG but not NG fibroblasts (Fig. 6). Such differences may reflect difference in the bio-behavior between the RG and NG fibroblasts in responding to CD44 treatment. Obviously, RG fibroblasts were activated and expressed significant higher levels of cell surface CD44 than that of NG fibroblasts. These results reiterate our previous observation that CD44-HA interaction facilitates mesenchymal cell adhesion and migration (*24*). In addition, HA in cultures sustained the survival of the intragraft fibroblasts in a serum-free conditioned medium. The RG fibroblasts exhibited a higher rate of cell proliferation during the first 3 days in culture and were able to maintain homeostatic cell survival for up to 8 days. The data are generally in agreement with previous reports that HA enhances mesenchymal cell proliferation, survival and differentiation *in vitro* (*31*, *37*, *38*). The results further support the notion that CD44–HA interaction is critically involved in the recruitment and growth of fibroblasts in allografts undergoing chronic rejection (*15*, *24*, *38*, *39*).

Up-regulation of CD44 expression by the intragraft fibroblasts is coupled with an up-regulation of HA synthesis. Intense staining of HA binding protein was present in the rejecting, but not in the non-rejecting heart allografts, nor in the native hearts. This notion is further supported by the findings that RG fibroblasts expressed high levels of HAS2 detected by qRT-PCR and increased significantly in HA secretion in conditioned media detected by competitive ELISA (Fig. 4). The data indicate that RG fibroblasts are an active producer of extracellular HA. Increases in HA deposition has been reported in heart allografts with acute rejection (*40*). The role of HA in chronic allograft rejection has yet to be clearly established. The current study suggests that extracellular HA in chronically rejecting allografts is pro-inflammatory and pro-fibrogenic. Accumulation of interstitial HA may enhance the ligand–receptor interaction and provides a niche to recruit fibroblasts into allografts. Extracellular HA can also interact with other members of the link family such as RHAMM to promote cell migration in a CD44-independent manner (*41*). In addition, HA fragments can induce macrophage production of several pro-inflammatory cytokines such as MIP-1α, MIP-1β and MCP-1 (*42*, *43*). The current data, together with other evidence, support our hypothesis that migration of fibroblasts of recipient origin to allografts is associated with interaction of CD44 and extracellular HA. CD44 was found to play a role in the reaction of vascular smooth muscle cells to arterial wall injury (*44*). An increase in HA deposition may also contribute to the aggregation of inflammatory infiltrates, especially macrophages in allografts, therefore, accelerating graft tissue inflammation (*45*). A reduction of the severity of chronic rejection in the LEW-to-F344 heart transplant model was achieved by intravenous infusion of a low molecular weight HA formulation, which may block CD44 molecules expressed by mononuclear cells (*46*).

In summary, CD44 gene expression by intragraft cardiac fibroblasts in association with HA-mediated cell activities has been studied in a rat model of chronic allograft rejection. A significant increase in CD44 expression by the RG fibroblasts was coupled with augmentation of extracellular HA synthesis in the heart allografts, indicating that CD44-HA interaction is critically involved in the pathogenesis of graft fibrosis. CD44 variant isoforms containing the v1 exon sequence were preferentially expressed by the rejecting graft fibroblasts. Somatic mutations were identified in the v1 sequence in the majority of the CD44v1 clones. Thus, CD44v1 isoforms may play a role, in a way similar to other documented v exons such as v3 or v6, to provide additional signalling for enhancing or suppressing the biological activities of intragraft fibroblasts. CD44 antagonism may affect multiple cellular activities mediated by the CD44-HA pathway, leading to alleviation of allograft fibrosis and chronic inflammation.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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