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ARTICLES

Absence of Cell-Surface Annexin V Is Accompanied by Defective Collagen Matrix Binding in the Swarm Rat Chondrosarcoma

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Abstract Annexin V has been characterized as a major collagen type II binding cell-surface component of normal chondrocytes and is also called anchorin CII in chondrogenic populations. Herein we present evidence that in vitro cultured Swarm rat chondrosarcoma cells are not capable of binding collagen type II in significant quantities to their surfaces, as compared to normal rat chondrocytes. This finding coincides with a deficiency of annexin V on the surface of these cells. A small quantity of an intracellular polypeptide could be detected which is immunologically cross-reactive with annexin V but displayed a mobility in SDS-PAGE of less than 34 kD compared to the M_r 36 kD of intact rat annexin V. By immunohistochemistry the protein could be localized in the cytoplasm of in vitro and in vivo grown tumor cells. By reverse transcription–polymerase chain reaction and Northern blot analysis, a regular-sized mRNA for annexin V could be detected in the chondrosarcoma cells that is expressed in only slightly lower quantities than in normal chondrocytes. Taken together, the data suggest a modified processing or turnover for annexin V in the chondrosarcoma excluding it from being a functionally active collagen type II binding protein. The findings support the hypothesis of cell-surface annexin V as a key component for the formation of the pericellular matrix of chondrocytes. J. Cell. Biochem. 65:131–144. © 1997 Wiley-Liss, Inc.

Key words: annexin V; extracellular matrix; cell surface; chondrosarcoma; chondrocytes

The annexins are a family of calcium- and phospholipid-binding proteins [for review see Moss, 1992]. At least one of the twenty members thus far described from this family can be found expressed in nearly every eukaryotic cell type. They are represented in a wide variety of species from primitive eukaryotes [Morgan and Fernández, 1995] to mammals [Barton et al., 1991]. As common as these proteins may be, no one clear function for all has been established.

One member of this protein family is annexin V (anx V) [Fernandez et al., 1988]. Annexin V has a M_r of 34–36 kD (depending on species) and contains four conserved 70-amino acid repeat domains found in all annexins [Morgan and Fernández, 1995]. A number of functional properties have been demonstrated for anx V, among them the binding of collagen [Mollenhauer and von der Mark, 1983; von der Mark et al., 1991; Kirsch and Pfäffle, 1992; Turnay et al., 1995].

In cartilage, annexins II, V, and VI have been detected [Mollenhauer and von der Mark, 1983; Genge et al., 1992; Suarez et al., 1993; Böhm et al., 1994]. Anx V in particular has been localized to the chondrocyte cell surface [Mollenhauer et al., 1984]. It was first named anchorin CII due to its location and proposed function of anchoring the chondrocyte to its extracellular matrix (ECM) [Mollenhauer et al., 1983] via

Abbreviations used: anx V, annexin V; DEPC, diethyl pyrocarbonate; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, hours; HRP, horseradish peroxidase; IgG, immunoglobulin G; NEM, N-ethylmaleimide; NRC, normal rat chondrocytes; PBS, phosphate buffered saline; rec, recombinant; RT-PCR, reverse transcription–polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRC, Swarm rat chondrosarcoma.

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binding to collagen type II, a protein which constitutes the major portion of all ECM molecules in most cartilage tissues. The protein and the cDNA sequences of anchorin CII have since been determined and found to be identical to those of anx V from other tissues [Fernandez et al., 1988; Böhm et al., 1994]. Cultured chondrocytes have high expression levels of anx V mRNA. However, until now no negative control system has been described which could be used to investigate the consequence of the absence of anx V from a chondrocyte cell surface. The present study of the Swarm rat chondrosarcoma (SRC) cells serves this purpose.

The SRC tumor [Choi et al., 1971] and its cultured sublines have been useful in the study of cartilage ECM macromolecules such as collagen types II, IX, and XI, the large proteoglycan aggrecan [Kimura et al., 1979; Fernandes et al., 1993], hyaluronan [Mason et al., 1982], cartilage oligomeric protein [Mörgelin et al., 1992], metalloproteinases, and a metalloproteinase inhibitor [Moses and Shing, 1994].

However, although SRC cells cultured in vitro produce all major molecular components of cartilage, the studies published thus far demonstrate that the collagen metabolism and deposition of ECM are seemingly impaired in the SRC [Fernandes et al., 1994]. One possible reason for this aberration could be a defect in expression and function of the cellular receptors for ECM. Herein we present evidence to link an impaired collagen type II binding to SCR cells with a defective expression of cell-surface anx V.

MATERIALS AND METHODS

Unless otherwise stated, all standard reagents and materials used were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available. All cell culture and molecular biology reagents were purchased from Life Technologies, Inc. (Grand Island, NY) unless otherwise specified.

Cell Culture

The SRC cells were originally obtained from the in vivo tumor as described by Hascall and Kimura [1981]. These cells were maintained in monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 25 ng/ml amphotericin B, and 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT) in 100 mm tissue culture dishes (Falcon; Becton-Dickinson & Co., Franklin Lakes, NJ). Cultures were passaged twice weekly using 0.25% trypsin in phosphate buffered saline (PBS) (150 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) and seeded at 2–5 $\times$ 10^6 cells/dish. Periodically, SRC cultures were examined and found to produce collagen type II, a marker for chondrocytic phenotype. Normal rat chondrocytes (NRC) were obtained from the sterna of newborn rat pups (aged 5–7 days) [Hagiwara et al., 1994]. Chondrocytes were dispersed in a sequential enzyme digestion with pronase (1 mg/ml DMEM, 1 h) (Calbiochem-Novabiochem Corp., La Jolla, CA), collagenase P (1 mg/ml DMEM with 5% FBS, 2 h) (Boehringer Mannheim Corp., Indianapolis, IN), and testicular hyaluronidase (0.5 mg/ml, 15 min) (Worthington Biochemical Corp., Freehold, NJ) adapted from Aydelotte and Kuettner [1988]. For most experiments cells were allowed to adhere to dishes in DMEM with 20% FBS for 2–4 h. Medium was exchanged for DMEM with 10% FBS for the remaining culture period.

Isolation of Collagen Types I, II, and III

Neutral salt extractions were used to isolate native collagens (with intact telopeptide extensions). Type I was prepared from fetal bovine amnion [Piez et al., 1963]. Type II was obtained from chick sternal cartilage as described by Wu et al. [1991]. Type III was isolated from lathyritic rat skin [Byers et al., 1974]. These collagens were purified to homogeneity as determined by SDS-PAGE. Purified collagens were labeled with ^{125}I by the chloramine T procedure [Greenwood et al., 1963]. Free isotope was removed by dialysis. Iodinated collagens were analyzed by 5% SDS-PAGE for quality control. Bands were visualized by autoradiography and then cut from the gel and measured for radioactivity in a gamma counter.

Collagen Binding Studies

The binding of radiolabeled collagen to NRC or SRC cells was performed as described [Mollenhauer et al., 1985]. After enzymatic dissociation of the ECM, chondrocytes were kept in medium containing hyaluronidase (500 μg/ml) and cycloheximide (25 μg/ml) according to Mollenhauer et al. [1984]. Cell suspensions were mixed in serum-free medium (DMEM with 25 mM HEPES, 2 mM EDTA, 0.1% BSA, pH 6.8) with 10^5 cpm iodinated collagens for 1 h at room

temperature. Cells were washed twice with medium and measured for bound radioactivity.

Antibodies

Polyclonal anti-anx V antiserum #8958 was raised against full-length anx V protein isolated from the cell membranes of chicken sternal cartilage as in Mollenhauer et al. [1984].

Polyclonal serum #9757 was raised against a 20 amino acid synthetic peptide of the N-terminal sequence of human anx V (Table I). The synthetic N-terminal peptide was coupled with bis (sulfosuccinimidyl) suberate to keyhole limpet hemocyanin according to manufacturer's protocols (Pierce, Rockford, IL) and used for immunization of rabbits as above [Mollenhauer et al., 1984]. For the antibody-binding studies described below, serum IgG was purified by affinity chromatography on Protein A columns according to the manufacturer's protocol (Pharmacia Biotech, Inc., Piscataway, NJ). Iodination of IgG with ^{125}I was performed by the chloramine T procedure as described above for the collagens [Greenwood et al., 1963].

Fluorescence-Activated Cell Sorting

Flow cytometric analysis of NRC and SRC surface proteins was performed as described by Mikecz et al. [1995]. Briefly, isolated chondrocytes were rinsed once in PBS, 5 mM EDTA, pH 7.3, resuspended in PBS, and incubated with antiserum #8958 or preimmune serum (1 μl/10^6 cells) on ice. Cells were washed and incubated with a biotinylated secondary antibody (Pierce) (1 μg/10^6 cells). Cells were washed again and incubated with phycoerythrin-conjugated streptavidin 20 min in the dark on ice, washed again, and finally fixed as a single cell suspension in

TABLE I. Protein Sequences for the N-Terminus of Annexin V*

Chicken	NH_2-M A K Y T R G T V T A F S P F D A R A D
Rat	NH_2-M A * * L R G T V T D F S G F D G R A D
Human	NH_2-M A Q V L R G T V T D F P G F D E R A D
Synthetic	NH_2-M A Q V L R G T V T D F P G F D E R A N

*Chicken [Fernandez et al., 1988], rat [Pepinsky et al., 1988; Imai and Kohsaka, 1995], and human [Pepinsky et al., 1988] anx V protein sequences for the first 20 residues and the synthetic peptide are depicted. Note that rat anx V does not contain positions 3 and 4 (denoted by * in the table).

1% formaldehyde. Immunofluorescence was detected by a FACSan flow cytometer (Becton-Dickinson & Co.) using Lysis II software.

Quantitative Antibody Binding Studies

Chondrosarcoma cell-surface anx V was further examined using radiolabeled IgG antibodies in the presence or absence of competing unlabeled IgG. Isolated SRC cells were incubated 1 h in medium containing testicular hyaluronidase (1 mg/ml). The cell suspensions (10^6 cells/sample) were mixed in 400 μl serum-free medium (DMEM with 25 mM HEPES, 0.1% BSA, pH 7.25, and 10% normal rabbit serum) with 10^5 cpm iodinated #8958, #9757, or control normal rabbit IgG in the presence or absence of competition by unlabeled #8958, #9757, or control IgG, respectively (1 mg IgG/ml final concentration) and incubated for 30 min at 4°C. The cells were washed twice with 10 ml ice-cold medium and measured for bound radioactivity.

Immunohistochemistry

Epitopes for anti-anx V serum #8958 were examined in fixed slices of normal rat tissue, SRC tumor pieces, and cultured SRC cells according to Chubinskaya et al. [1996]. The in vivo–grown SRC tumor pieces were a gift of Dr. Cheryl Knudson (Departments of Biochemistry and Pathology, Rush Medical College). Tissue specimens were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, cut into 5 μm sections, and mounted on gelatin-coated glass slides. Prior to immunostaining, the sections were treated with chondroitinase ABC (ICN Biomedicals, Inc., Costa Mesa, CA) (at 10 mU/ml in 100 mM Tris, 50 mM sodium acetate, pH 6.5, 37°C, 90 min). Unspecific immunoglobin binding was blocked with 1% normal goat serum in PBS/2% BSA. Immunostaining was performed in PBS/BSA with a 1:100 dilution of antiserum #8958 at 4°C for 16 h. The slides were washed with PBS/BSA and incubated with goat antirabbit horseradish peroxidase-conjugated second antibody for 2 h. After rinsing, the staining was developed with diaminobenzidine. The slides were again dehydrated and mounted with a cover slip.

In Situ Hybridization

A 29 mer antisense oligonucleotide probe (5′-CCA-GAG-AAG-TCA-GTC-ACG-GTG-CCT-CTG-AG-3′) was designed with a sequence

complementary to base pairs 75–103 of rat anx V mRNA [Pepinsky et al., 1988]. The specificity and sequence homology of this probe was compared with sequence data available from the European Molecular Biology Laboratories/Genbank DDB data base. The gel-purified oligonucleotide probe was 3′-end-labeled with 5′-[α-thiol-^{35}S]-deoxycitidine triphosphate using terminal deoxynucleotidyl transferase (New England Nuclear, Wilmington, DE). The radiolabeled probe was hybridized to the histological sections as previously described [Sandell et al., 1991]. Autoradiographs were used to visualize mRNA probe hybridization by exposing sections to photographic emulsion (Kodak NTB2; Eastman Kodak Co., Rochester, NY) for 3–7 days at 4°C. The emulsion was developed in D19 solution (Eastman Kodak) diluted 1:1 with distilled water at 16°C. Sections were counterstained with cresyl violet acetate and sealed with a coverslip.

Enrichment of Annexin V

Soluble and insoluble cellular proteins were separately examined for anx V. Monolayers of SRC cultured for 72 or 96 h were rinsed twice in PBS. The cell layers were extracted in ice-cold buffer (20 mM Tris, 8% NaCl, 1 mM NEM, 1 mM PMSF, 10 mM benzamidine HCl, pH 7.5, with 1 mM $CaCl_2$) with brief ultrasonication [Böhm et al., 1994]. The extracts were centrifuged at 100,000*g*, 60 min, at 4°C. Samples of supernatants with soluble protein and pellets with membrane-bound and otherwise insoluble proteins were analyzed by SDS-PAGE and Western blot.

Further enrichment of anx V was achieved by anion exchange chromatography. The soluble proteins were dialyzed against 2 M urea, 20 mM Tris, 2.5 mM EDTA, pH 7.8. Approximately 5 mg protein were applied to a Mono Q column (Pharmacia Biotech, Inc.) equilibrated in the buffer above as described by Böhm et al. [1994]. The breakthrough and 0.5 ml fractions were collected during an elution gradient of 0–1.0 M NaCl with a total gradient volume of 15 ml at a flow rate of 0.5 ml/min. The fractions were analyzed by SDS-PAGE and Western blot.

Production of Recombinant (rec) Human Annexin V

The pRK6 plasmid strain in *E. coli* TG1 was purchased from the American Type Culture Collection (Rockville, MD). The insert cDNA of this plasmid is the human placenta anx V [Ritty et al., 1991]. The rec human anx V protein was harvested from bacteria cultured as described by Kaplan et al. [1988]. Briefly, the bacteria were cultured in Luria broth with ampicillin overnight. These cells were sonicated and centrifuged. Aliquots of the supernatant were added to SDS-PAGE sample buffer.

Isolation of Chicken Sternal Membranes

Membranes were isolated from chicken cartilage as previously described [Böhm et al., 1994; Mollenhauer and von der Mark, 1983]. Briefly, the cartilage was homogenized in 10 mM Tris/HCl, pH 7.2, containing 8.5% (w/w) sucrose, 2 mM EDTA, and protease inhibitors. The homogenate was subjected to high speed centrifugation (50,000*g* for 2 h). The resulting pellet was resuspended in the same buffer and purified over a discontinuous sucrose density gradient (40% and 17% (w/w) sucrose) in an SW-40 rotor (Beckman, Fullerton, CA) for 1.5 h at 100,000 rpm and 4°C. The plasma membrane proteins were enriched at the 17%/40% sucrose interface.

Western Blotting

Following the methods of Towbin et al. [1979], samples (50 μg) were separated by SDS-PAGE and electroblotted to nitrocellulose. The anx V–specific bands were identified with antisera #8958 and #9757. The bound primary antibodies were detected with HRP-conjugated goat antirabbit IgG and developed with 4-chloro-1-naphthol in PBS, pH 6.0.

Isolation of Ribonucleic Acid

Total RNA was isolated from monolayer cultures of NRC and SRC cells. Primary chondrocytes isolated as above (see Cell Culture) were cultured for 48 h in 100 mm tissue culture dishes (10^7 cells/dish) in DMEM with 10% FBS. Cultures were extracted with Trizol (Life Technologies, Inc.) (guanidine isothiocyanate and phenol extraction procedure of Chomczynski and Sacchi [1987]). The quantity and quality of RNA were determined by ultraviolet absorbance and electrophoresis as described by Sambrook et al. [1989].

Polymerase Chain Reaction (PCR)

Isolated total RNA samples from NRC and SRC cells were used in reverse transcription to

prepare cDNAs which were then amplified by PCR [Erlich et al., 1991]. Rat anx V–specific primers were selected using PCGENE computer software and synthesized by Research Genetics, Inc. (Huntsville, AL). See Table II for a list of primer pairs used. Superscript Rnase H-reverse transcriptase (Life Technologies, Inc.) was used for first-strand cDNA synthesis. A portion of the resulting cDNA solution was carried to PCR using both minus and plus primers for 35 cycles. Products were purified by filtration and by electrophoresis in agarose gels. Manufacturer's protocols were observed (Life Technologies, Inc.).

RNA Electrophoresis and Northern Blotting

The size and relative quantity of the anx V–specific messages expressed by NRC and SRC cells were examined by Northern blotting and hybridization. Isolated RNA was ethanol-precipitated, washed, dried, and resuspended in sample buffer (50% formamide, 7% formaldehyde, 1× MOPS (MOPS: 20 mM 3-[N-morpholino] propanesulfonic acid, 50 mM sodium acetate, 1 mM EDTA, pH 7, 0.2% bromophenol blue). Samples containing 5 µg RNA were denatured 15 min at 65°C, chilled on ice, and loaded into the wells of a 1.5% agarose gel (containing 1× MOPS and 0.66 M formaldehyde, final concentration). Separation was performed in 1× MOPS at 55 V for 4 h. Formaldehyde was removed by soaking the gel in DEPC-treated water and then 20× SSPE (3 M NaCl, 0.2 M NaH_2PO_4-H_2O, 0.02 M EDTA, pH 7.4) prior to overnight capillary transfer in 10× SSPE to nylon membranes. The membranes were baked at 80°C for 2 h to immobilize RNA.

RNA Slot Blot Preparation

The quantity of anx V–specific message was examined by slot blot analysis. Serial dilutions of total RNA (1:2 dilutions, 2.0 to 0.125 µg) were prepared in 50% formamide, 7% formaldehyde, and 1× SSC and denatured for 15 min at 65°C. These samples were applied to nylon membranes using a standard vacuum apparatus. Sample wells were washed twice with 10× SSC and dried. Membranes were baked at 80°C for 2 h to immobilize RNA.

Preparation of Nucleic Acid Hybridization Probes

The anx V probe was created by reverse transcription–polymerase chain reaction (RT-PCR) of NRC RNA using the rat-specific primers P61 and M537 (Table II). The plus strand primer P61 targets the start AUG codon site. The minus strand primer M537 is targeted from 467–491 bases 3′ of the start site. The double-stranded DNA was sequenced and found to be identical to the published rat anx V sequence [Pepinsky et al., 1988; Imai and Kohsaka, 1995]. The control hybridization probe was prepared by PCR using a plasmid containing human GAPDH sequence (Life Technologies, Inc.). Double-stranded DNA probes were labeled by random priming using α-^{32}P-dCTP (Amersham Life Sciences, Inc., Arlington Heights, IL) and the Klenow fragment of DNA polymerase (Life Technologies, Inc.).

Hybridization

Total RNA bound to the nylon membranes was hybridized to radiolabeled anx V–specific DNA probes. Membranes were treated over-

TABLE II. Primers Used for RT-PCR and for Cycle Sequencing*

	Primer	Primer sequence (5′ to 3′)	Location
Template	P61	ACAGCATCATGGCTCTCAGAGGC	61–83
Pair (I)	M537	TGCAGTGTCAGGGTCTCTATTGGC	537–560
Template	P174	ATCCTGAACCTGTTGACAGCCCG	174–196
Pair (II)	M853	CTCTGATGAGGGTGTGATCGTCCG	853–876
Template	P562	TTGATGATGCTCAAGTTGAACTGG	562–585
Pair (III)	M1083	TGAACGTAAGTAGGCGTGGTGC	1,083–1,104
Template	P739	ACCGAGAGACCTCAGGGAACTTGG	739–762
Pair (IV)	M1156	CTGCTTTCATTCGAGACAGCTTTGG	1,156–1,180

*Rat anx V–specific primers were used for RT-PCR and for cycle sequencing. The location numbers are according to Pepinsky et al. [1988] in which the start codon appears at 69 and the termination codon at 1028. M, minus strand primer; P, plus strand primer. Together these templates include the entire sequence of the coding region for rat anx V.

night at 48°C (50% formamide, 5× Denhardt's solution (United States Biochemical, Cleveland, OH) 10% dextran sulfate, 5× SSPE, 1% SDS). Hybridization proceeded overnight with approximately 25 ng of denatured DNA probe in fresh buffer (same composition as prehybridization solution) at 48°C in sealed glass bottles. The membranes were washed at 50°C twice in 2× SSPE, 0.1% SDS for 15 min, once in 1× SSPE, 0.1% SDS for 60 min, and once in 0.1× SSPE, 0.1% for 15 min. The washed membranes were rinsed in 2× SSPE. The bound radioactivity was detected with autoradiography or autoprocessing (STORM; Molecular Dynamics, Inc., Sunnyvale, CA). Membranes were stripped of the annealed anx V probe by incubation at 75°C for 1 h in 60% formamide, 50 mM Tris HCl, pH 7.5–8.0, 1% SDS and hybridized to the GAPDH probe.

Sequencing of SRC Annexin V cDNA

Isolated total RNA from cultured SRC was reversely transcribed with primer M1156. Purified templates, double-stranded segments of the SRC anx V cDNA made by PCR using primers listed in Table II as templates I–IV, were sequenced by cycle sequencing, a variation of the dideoxy-chain termination method [Sanger, 1977] according to the manufacturer's directions (Life Technologies, Inc.). Denatured sequencing products were resolved by electrophoresis on high resolution, 8% polyacrylamide–7 M urea gels in the presence of 1× TBE buffer (100 mM Tris, 90 mM boric acid, 1.0 mM EDTA, pH 8.33). Maximum resolution was achieved by electrophoresis of identical sequencing products for varying time periods (30 min or 1, 2, or 4 h) enabling sequencing of the entire templates. The gels were dried and analyzed by autoradiography. Each autoradiogram was read independently by two persons. Results were compared to published sequences.

RESULTS

Collagen Binding Studies

Since the irregularities of collagen matrix formation are a prominent feature in a subline of SRC [Fernandes et al., 1993, 1994], the ability of SRC cells to bind collagen type II to their surfaces was examined and compared to that of normal chondrocytes. Tropocollagens with intact telopeptides were used since it is likely the interaction between anx V and collagen type II requires the presence of the collagen N- and/or C-terminal telopeptide region [Mollenhauer and van der Mark, 1983; Mollenhauer et al., 1985]. The cells were stripped of their ECM with collagenase and testicular hyaluronidase and incubated with radiolabeled collagens. Unbound collagen was separated from the cells by centrifugation at the end of the incubation period. Considering the specific activity of the radiolabeled collagen, one million NRC cells were able to bind specifically to collagen type II (151 ng) but bound collagen types I and III less efficiently at 0.01 ng (Table III). The binding of one million SRC cells to collagen types I, II, and III was 0.02, 3.4, and 0.01 ng, respectively. In other words, one SRC cell bound about 1.2×10^{-20} moles of collagen type II, while one NRC cell bound nearly fifty times more collagen type II (5.3×10^{-19} moles).

Flow Cytometric Analysis and Binding of I-125–Labeled Antibody

To determine if a specific loss of surface-located anx V caused the inability of SRC to bind collagen, their cell surfaces were examined using flow cytometry as reported in Figure 1. Matrix-free cells in suspension were mixed without (Fig. 1A,B) or with the anx V–specific polyclonal antiserum #8958 (Fig. 1C,D). All incubations were at 4°C to prevent the penetration of antibody into the live cells and to inhibit cell membrane turnover. Approximately 80% of the NRC cells showed positive fluoresence compared to 13% of the SRC cells. However, about 12% of the SRC cells treated with control IgG were also beyond the control levels established with the NRC cells.

To determine what portion of the binding of #8958 to the SRC cells detected by flow

TABLE III. Collagen Binding to NRC and SRC Cells*

Cell type	Collagen type	Mean mol per 10^6 cells	Mean ng per 10^6 cells
NRC	I	5.5×10^{-17}	0.01
	II	5.2×10^{-13}	150.9
	III	1.8×10^{-17}	0.01
SRC	I	9.0×10^{-17}	0.02
	II	1.2×10^{-14}	3.4
	III	1.8×10^{-17}	0.01

*The table displays the quantities of collagen types I, II, and III bound to NRC or SRC cells. Cells were incubated 1 h at room temperature with radiolabeled collagen. Collagen-bound quantities were calculated using the specific activity of ^{125}I collagen.

cytometry was caused by specific binding to surface-located anx V or by unspecific IgG adsorption, a competition assay was performed. In this assay, unlabeled IgG competed with radiolabeled IgG (Table IV). No specific binding of anti-anx V could be detected on SRC cells.

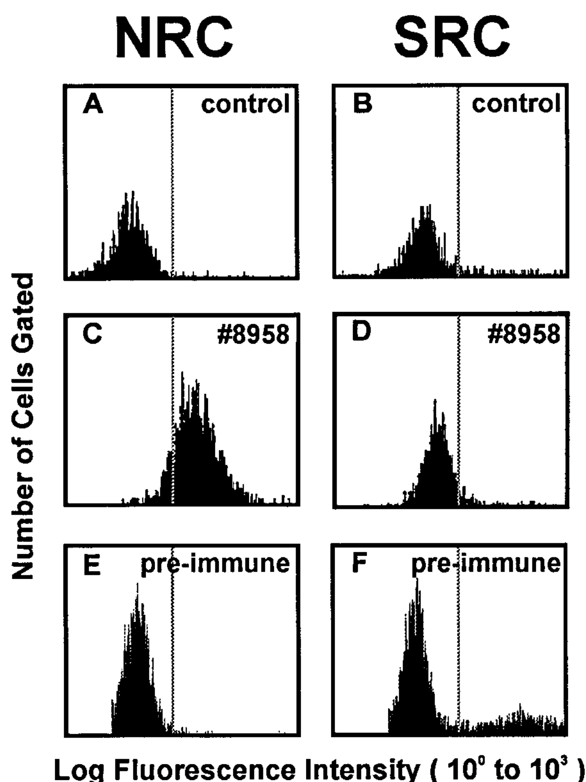

Fig. 1. Flow cytometric analysis for anx V surface expression. Matrix-free cells of NRC (A,C,E) and SRC (B,D,F) were incubated with antichicken anx V antiserum (c,d), washed, and identified with biotinylated second antibody and streptavidin-conjugated phycoerythrin. For controls (a) and (c) no primary antiserum was used; for controls (e) and (f) preimmune serum served as primary antiserum. Immunostaining was detected by a FACSan flow cytometer.

Immunohistochemistry

To examine the presence of immunoreactive anx V in subcellular locations other than the surface, histological specimens of SRC cells in monolayer and of in vivo–grown tumor pieces were examined (Fig. 2). Slices of normal rat cartilage were also examined. The articular chondrocytes of the rat tibia showed significant intracellular immunostaining for anx V. The cells of the rat sterna showed intracellular staining of the cytoplasm and intense staining of the cell borders and lacunar walls. The tumor cells grown subcutaneously in vivo in rats showed some immunoreactivity. Significant immunostaining was evident in the connective tissue septae from the host animal invading the tumor tissue. The SRC monolayer culture samples showed variable staining. Most SRC cells, however, were negative for anx V staining.

In Situ Hybridization

In situ hybridization experiments were performed on sections of normal rat sternum and on in vitro–grown SRC cells using a probe specific for the N-terminal coding region of rat anx V (Fig. 3). In both the normal tissue and the tumor line, all cells displayed positive signals.

Western Blot Analysis of Annexin V From SRC Cultures

In order to examine the nature of the positive immunostaining in the histological investigations, NRC and SRC cell extracts were separated by electrophoresis and analyzed by Western blotting. Initially, whole cell extracts of SRC were examined for immunoreactivity to antichicken anx V (#8958). However, no bands near the expected molecular weight for anx V could be identified (data not shown). To enrich SRC samples for annexins, two approaches were

TABLE IV. Binding of ^{125}I-Labeled Immunoglobins to SRC Cells*

Iodinated IgG	Unlabeled inhibitor (1 mg/ml)	Percent radioactivity bound	Standard deviation	Paired t-test[a]
Preimmune	None	7.0	0.3	—
Preimmune	Preimmune	4.9	0.9	$P = 0.004$
#8985	None	7.6	1.6	—
#8958	#8958	7.1	1.9	ns ($P = 0.62$)

*Isolated chondrocytes were mixed with iodinated anti-anx V #8958 IgG or normal IgG with or without competing unlabeled IgG for 30 min in the presence of DMEM with 10% normal rabbit serum. The experiments were performed in quadruplicate.
[a]Student's t-test of difference in bound radioactivity between the groups with and those without the corresponding inhibitor. ns, statistically not significant.

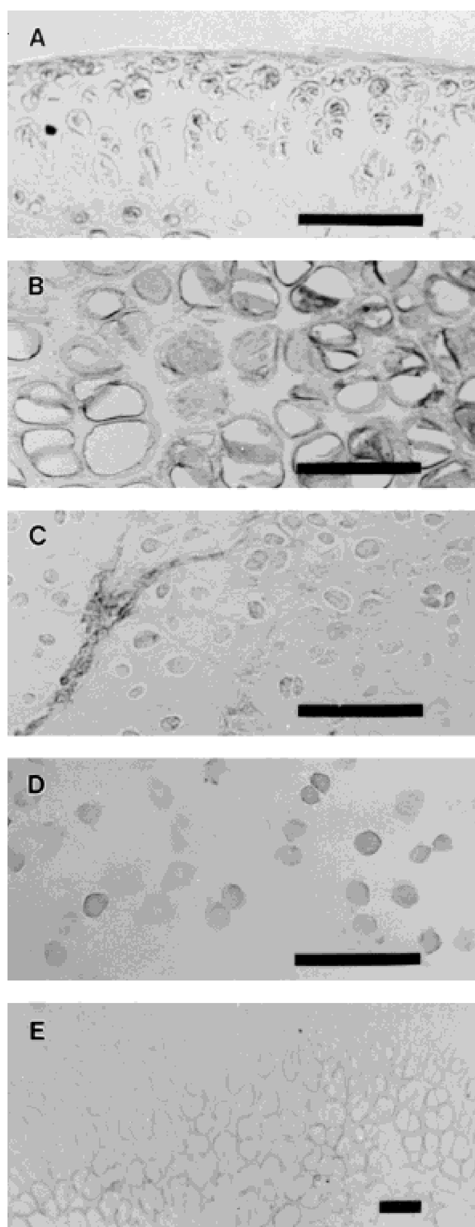

Fig. 2. Immunohistochemistry to locate anx V epitopes in normal rat tissues and SRC. Paraffin sections (7 μm) of normal rat tibia (A), sterna (B), and SRC tumor (C) and fixed monolayer cultures of SRC (D) were incubated with antichicken anx V antiserum (#8958). Bound antibody was detected with goat antirabbit IgG conjugated to horseradish peroxidase and developed with diaminobenzidine substrate. Control (E) rat sterna tissue was not incubated with #8958. **A:** Normal rat tibia with intense staining of the cells. **B:** Normal rat sternal chondrocytes from the middle region with cell-surface and cytoplasmic staining and staining of lacunar walls. **C:** SRC in vivo tumor section with low general staining, some cellular staining of groups of tumor cells, and intense staining of host connective tissue. **D:** Monolayer of cultured SRC in which only small groups of cells are stained. **E:** Preimmune control. Bars = 50 μm.

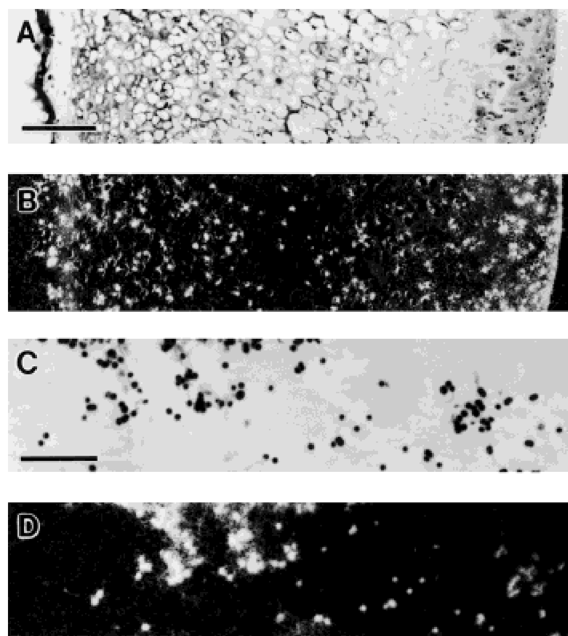

Fig. 3. In situ hybridization of rat anx V probe to the nuclei of newborn rat sternum and SRC cells in monolayer culture. The sequence of the probe was 5′-CTC-AGA-GGC-ACC-GTG-ACT-GAC-TTC-TCT-GG-3′. Newborn rat sternum: bright field (**A**) and dark field (**B**). SRC in monolayer culture: bright field (**C**) and dark field (**D**). Bars = 250 μg.

used: (1) separation of soluble from insoluble proteins and (2) ion exchange chromatography. In normal chondrocytes, the majority of anx V is typically found in the cell membrane. The centrifugation pellets (insoluble proteins) and supernatants (soluble protein fractions) were electrophoretically separated and either stained for protein or electroblotted and incubated with either #8958 or #9757. Antibody #9757 was used because of the high homology of the human sequence to the rat sequence in the N-terminal 20-amino acids of anx V (Table I). As positive controls for the antibodies, extracts of normal chick sternum membranes (for #8959) and extracts of bacterial lysates with rec human anx V (for #9757) were included.

The antichicken anx V #8958 identified the chick anx V (Fig. 4, left side, lane 1). It also identified rec human anx V from the bacterial lysate and, in addition, some fragments (Fig. 4, left side, lane 2). The antiserum raised against the 20 amino acid synthetic N-terminal peptide (#9757) showed weak immunoreactivity to chicken anx V (Fig. 4, lane 1 from right) but did have strong immunoreactivity to the rec human anx V. The normal rat anx V had an apparent molecular weight of about 36 kilodal-

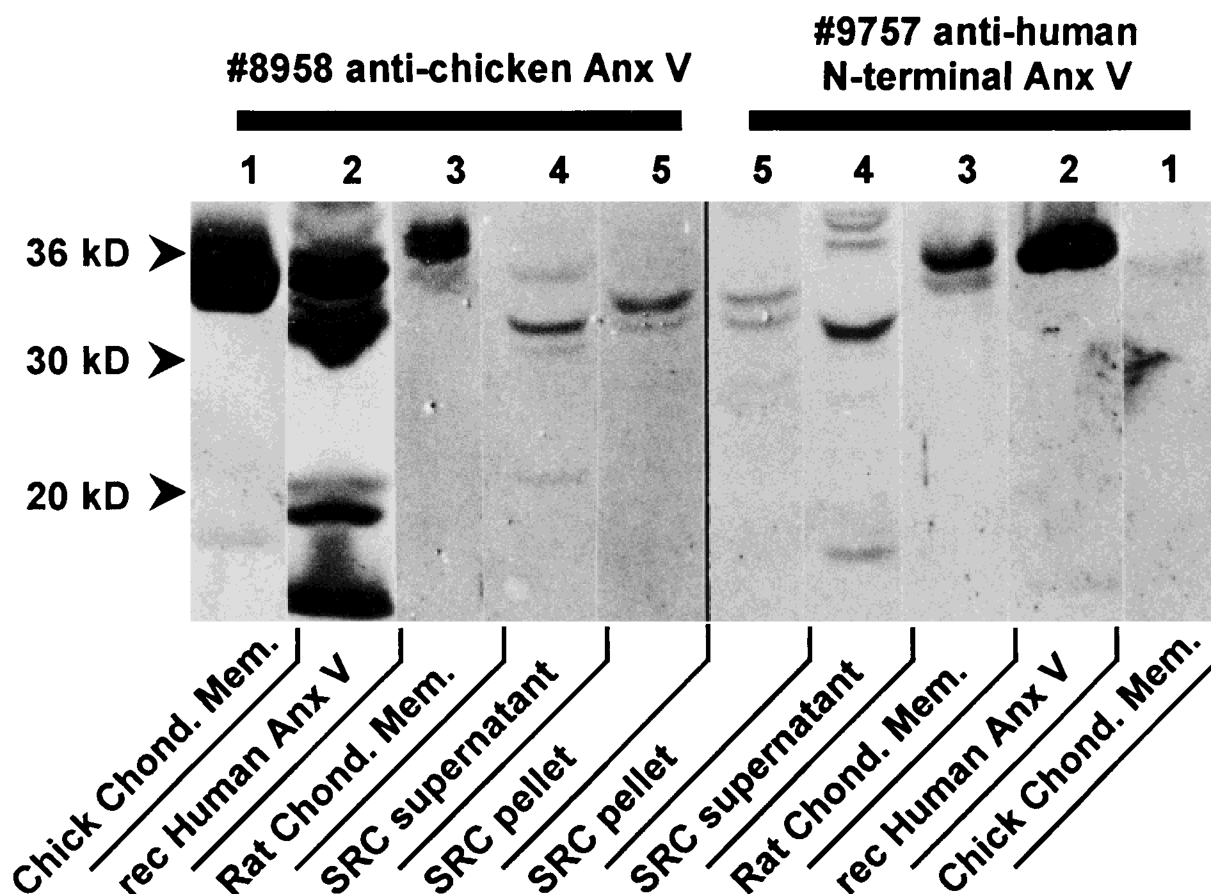

Fig. 4. Electrophoretic separation and Western blot analysis of extracts of normal tissues and SRC monolayers. **Left side:** Western blot analysis using polyclonal antichicken (full-length) anx V antiserum (#8958). **Right side:** Western blot analysis using polyclonal antihuman synthetic N-terminus anx V. (#9757). Samples (50 µg for lanes 1, 3, 4, 5) are arranged in mirror fashion. *Lane 1:* Chick chondrocyte membranes. *Lane 2:* 10 µg rec human anx V. *Lane 3:* Normal rat chondrocyte membranes. *Lane 4:* SRC supernatant. *Lane 5:* SRC pellet. The SRC samples were prepared by extraction and high-speed centrifugation of SRC cell monolayers.

tons and was recognized by both antisera (#9858 and #9757) (Fig. 4, left and right sides, lane 3).

The extracts from SRC (Fig. 4, lanes 4, 5) displayed peptides ranging from 35 to 19 kilodaltons but none with the expected molecular weight. There were no bands with the same migration as the anx V from NRC. More polypeptide bands were identified and were more reactive in the supernatant (soluble protein fractions) (Fig. 4, lane 4) than in the pellet (insoluble protein fractions) (Fig. 4, lane 5), suggesting a larger amount of immunoreactive peptides in the cytosol over the membranes.

There were also differences in SRC protein recognition by the two sera. Some bands which were recognized by #8958 (generated against full-length anx V), such as the 35 kD band of the supernatant extracts (Fig. 4, left side, lane 4), did not appear with #9757, which suggested the absence of the N-terminal portion of anx V. Other peptides such as the 19 kD band were identified by #9757 (Fig. 4, right side, lane 4), indicating these peptides contained N-terminal epitopes. Further purification of normal chondrocyte extracts by anion ion exchange chromatography would normally enrich anx V [Pepinsky et al., 1988; Böhm et al., 1994]. However, only the fragmented anx V peptides described above were recognized by antisera #8958 and #9757 during Western blotting of chromatography fractions (data not shown).

Expression of the SRC Annexin V Message

The results of the protein analysis suggested mutated genes, alternative splicing, or premature degradation of the primary translation product in SRC cells. To identify alternative splicing and expression levels, we examined the

anx V messenger RNA produced by SRC cells in culture by the following three techniques: RT-PCR, Northern and RNA slot blot analysis, and sequencing of anx V cDNAs produced directly from total RNA.

The sizes of RT-PCR products of SRC samples were compared to those of NRC by agarose gel electrophoresis. For all primer pairs used, the PRC products of both NRC and SRC exhibited identical mobilities (not shown).

To measure SRC anx V mRNA size, total RNA samples from SRC cultures (suspension or monolayer) and total RNA from NRC cultured in monolayer were separated by electrophoresis. Northern analysis was performed using a radiolabeled DNA probe prepared from RT-PCR of normal rat RNA using a primer pair targeted to the N-terminal half of the rat anx V sequence. Autoradiography showed hybridized species of identical size in SRC and NRC (Fig. 5). For RNA loading control, the membrane was stripped and hybridized to a control GAPDH probe. Quantification and integration of the radioactivity of these Northern blots in the area of the specific RNAs showed that, after adjusting for loading differences by normalization to GAPDH hybridization, the RNA species from SRC cells cultured in monolayer hybridized about the same amount of anx V probe as the RNA from NRC cultured in monolayer (Table V).

In slot blot analyses, serial dilutions of total RNA from monolayer cultures of SRC and NRC were applied to membranes, fixed, and hybridized to the anx V probe as above. In three independent experiments and over a range of 1 μg to 62.5 ng, the NRC RNA hybridized more than twice the anx V probe as SRC RNA. Both the NRC and the SRC RNA hybridized about the same amount of GAPDH control probe (data not shown).

The cDNA of SRC anx V was sequenced to identify any gene mutations which might produce alternative initiation and termination signals in SRC transcripts resulting in truncated proteins. Primer pairs I, II, III, and IV of Table II were used to produce overlapping templates, as shown in Figure 6. Primers P61, P174, P562, and P739 were used to sequence the cDNA plus strand. Primers M537, M853, M1083, and M1156 were used to sequence the cDNA minus strand. The cDNA of the entire mRNA for SRC anx V was sequenced. In all, no deviation from published sequences was found in the coding region of SRC anx V (data not shown).

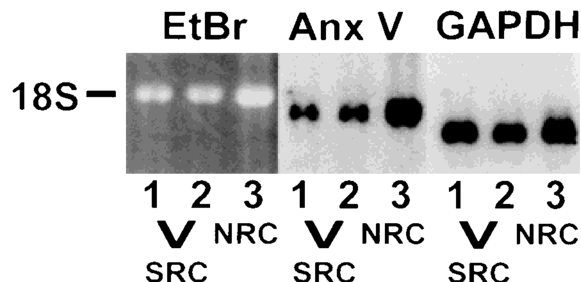

Fig. 5. Northern analysis of SRC mRNA. **Left:** Electrophoresis of 5 μg total RNA in a 1% agarose formaldehyde gel. Nucleic acid was visualized with ethidium bromide. **Center:** RNA was blotted to nylon membrane and hybridized to ^{32}P-labeled anx V probe to detect rat annexin V message. **Right:** RNA was hybridized to ^{32}P-labeled GAPDH probe for RNA loading control. In all images the lanes are as follows: *lane 1*, total RNA from SRC cells cultured in suspension; *lane 2*, total RNA from SRC cells cultured in monolayer; *lane 3*, total RNA from NRC cultured in monolayer.

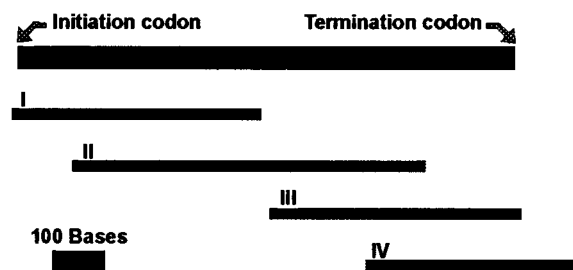

Fig. 6. Scale diagram of cDNA templates used for sequencing anx V mRNA from SRC cells. Template numbers (I, II, III, IV) correspond to template primer pairs noted in Table II.

TABLE V. Quantification of Northern Blot*

Total RNA from cultures	Anx V (cpm)	GAPDH (cpm)	Normalized amount of Anx V
SRC suspension	135	801	0.5
SRC monolayer	187	571	1.0
NRC monolayer	285	875	1.0

*The Northern blot depicted in Fig. 5 was analyzed for the quantities of hybridized radioactivity for both the anx V and the GAPDH probes.

DISCUSSION

The role of anx V in cartilage as a collagen-binding protein has been investigated in normal chondrocytes [Mollenhauer and von der Mark, 1983, 1984; von der Mark et al., 1991] and was found to depend upon the cell-surface location of the protein in chondrocytes. Therefore, the protein was named anchorin CII. We have shown that the SRC cells did carry the gene for anx V and were able to produce anx V mRNA. By RT-PCR analysis, Northern blotting

and hybridization, and cDNA sequencing, we proved this mRNA to be identical to normal rat chondrocyte mRNA in size and sequence. The SRC cells were not, however, able to produce the final translation product in the appropriate quantities, as an intact structure, and, in particular, in a location to function as a collagen type II receptor at the cell surface. SRC cells did accumulate small quantities of peptides as soluble cytoplasmic components with apparent molecular weights lower than anx V, possibly an indication of premature digestion of the primary translation product.

Northern blot analysis showed that the SRC cells produce anx V mRNA of the expected size for rat anx V (1.6 to 1.7 kilobases [Fernandez et al., 1988; Karube et al., 1995]). Similar levels of anx V mRNA expression were found in both NRC and SRC cells, with a slight reduction (up to 50%) in SRC cells, depending upon culture conditions. This could have accounted, in part, for lower levels of total anx V–related peptides identified in SRC extracts but could not account for the lower molecular weight of these peptides.

Karube et al. [1995] have reported that cervical and endometrial carcinoma cells have a decreased (transcriptional) expression of anx V when compared to their normal counterparts. Mohiti et al. [1995] have reported the opposite for osteosarcoma. Their MG-63 human osteosarcoma cell line expressed three times more anx V protein than normal osteoblasts. In other words, the different levels of mRNA and/or protein expression could not be linked to a phenotypic pattern of these tumor lines.

We have used RT-PCR to produce four overlapping templates of SRC anx V cDNA in order to identify possible gene mutations which could result in truncated proteins. These templates were 500–800 bases each and together encompassed the cDNA length of 1,120 bases, including the entire coding region. Examination by electrophoresis showed the templates to be of expected size. Sequencing of the templates showed no variance from normal rat anx V sequence from the start codon to beyond the termination codon. There were no unusual splicing events or detectable mutations.

We have conducted an investigation of the expression of anx V message in SRC on the individual cell level by in situ hybridization using an oligonucleotide targeted to the N-terminal coding region of rat anx V mRNA. Sections of normal rat sterna showed hybridization of the probe to chondrocyte nuclei throughout the tissue. In SRC cells cultured in monolayer, there was a positive hybridization signal in each cell inspected. This indicated that all or most SRC cells were producing this message. Therefore, it is unlikely that subpopulations of clonal groups produced extremely low levels of message or were completely void of anx V mRNA.

Despite the apparently normal transcription of message, only barely detectable amounts of anx V protein could be identified by cell-surface analysis and Western blot analysis. There was antigen recognition by the anti-anx V antibody in immunohistochemistry of SRC tumor and cell-culture samples. However, the antigens were restricted almost entirely to the cytosolic regions of the SRC cells since no cell-surface-located anx V could be detected by either flow cytometry or binding studies with iodinated antibody except for some nonspecific IgG binding that may be related to Fc receptors [Takagi and Jasin, 1992; Saura et al., 1993]. These results did not vary with the extent of treatment of the cells with hyaluronidase or the time allowed for recovery of the surface structures prior to the flow cytometrical evaluation or the binding study with iodinated antibody. The immunohistochemical sections of normal rat tissues and the flow cytometric data of NRC indicated a pattern expected for normal chondrocytes as published by Mollenhauer et al. [1984] and Böhm et al. [1994]. All normal rat chondrocytes appeared to produce both anx V mRNA and protein including cell-surface-deposited anx V. Taken together, the data suggest a reduction of anx V expression on the cell surface of SRC cells below the limits of detection.

The majority of anx V peptides in SRC extracts were detected by Western blots in the soluble protein fraction. This confirmed the cytosolic staining of SRC seen by immunohistochemistry. However, the peptides showed great variability in their molecular weights, most likely indicating various stages of intracellular degradation. Suarez et al. [1993] reported faint bands of small molecular weight peptides identified by anti-annexin I, II, V, and VI antibodies in rat osteoblast extracts. Kristensen et al. [1993] also reported identification of an additional anx V polypeptide with lower molecular weight in murine intestine extracts.

It is not likely that peptidases have selectively destroyed the N-terminus of SRC anx V

because antiserum #9757 (directed against the N-terminal domain of anx V) did identify multiple bands in the Western blot. Some of these small peptides, however, were only identified by one of the two sera, not both. Taken together, this pattern suggests an "unspecific" cleavage pattern during intracellular degradation of anx V in SRC.

Ion exchange chromatography is a standard technique for the purification of anx V [Seaton et al., 1990; Böhm et al., 1994]. With this additional purification step, we were able to clearly identify a number of anx V fragments in various chromatographic fractions. Again, we estimated that the total amount of anx V fragments from the SRC cells was less than 5% that of normal chondrocytes.

The reason for the destabilization of anx V in SRC cells was not clear. A point mutation in the start codon or consensus sequence could have led to a truncated translation product. A peptide could have been translated from a downstream start codon provided the eukaryotic version of the Shine-Delgarno consensus sequence was in the right context [Rhoads, 1991]. For the rat anx V mRNA, the first alternative in frame AUG is 72 bases beyond the normal start codon. Translation beginning at this point would have resulted in a peptide missing the first 24 amino acids. However, such a peptide would not likely have been recognized by antiserum #9757 which was raised against only the first 20 residues.

It is possible that a portion of one or more of the four conserved 70-amino acid repeat domains was misfolded, leading to premature degradation. The importance of the repeat domains was illustrated in the crystal-structure analysis by Huber et al. [1990]. The resulting model of the human anx V structure suggested that the calcium-binding domains I, II, and IV (domain III in rat anx V also binds calcium [Concha et al., 1993]) attach the protein's convex face to the phospholipid membrane. Lu et al. [1995] supported this hypothesis with a model of calcium- and phospholipid-binding kinetics. A defect in the three-dimensional structure of one or more of the domains of the truncated polypeptides could explain the incapacity of these peptides for a stable association with plasma membranes.

An alternative explanation is focused not on the primary translation product but on the translocation of anx V. The reported cDNAs for the annexin family did not provide for a signal peptide nor a classic transmembrane sequence [van Heerde et al., 1995]. Exactly how the completed protein is transported to and through the cell membrane was not explained. Mohiti et al. [1995] reported that increases in intracellular calcium were followed by an increase in anx V immunolocalized to the nuclear envelope. Christmas et al. [1991] have examined the secretion of annexin I and suggested that a type of vesicularization could take place which would be analogous to matrix vesicle formation by chondrocytes [Genge et al., 1989, 1990].

Perhaps the SRC cells did not contain one or more of the particular elements required for this unknown transport mechanism. If membrane insertion provided stability to normal chondrocyte anx V, then a transport defect could have resulted in anx V intracellular degradation despite proper transcription and translation and, ultimately, have led to the described defect in collagen binding. On the other hand, a general defect of the cell membrane metabolism can be reasonably excluded since such a defect would render SRC cells unviable. The SRC cells provided an opportunity to study the consequence of defective expression of anx V to the chondrocyte and its ECM.

Collagen-binding integrins (α_1, α_2, α_3, α_5, α_v, β_1, β_3, and β_5 chains) are expressed in vivo and in cultured normal chondrocytes and may in part provide for additional binding sites for collagen [Dürr et al., 1993; Woods et al., 1994; Loeser et al., 1995]. However, little is known about integrin expression in SRC. By Western blotting of membrane fractions we detected intact β_1 chains (Dürr and Mollenhauer, unpublished data). To what extent integrins in the SRC contribute to the binding of the collagenous matrix remains to be studied. The very low levels of collagen binding in the SRC compared to normal chondrocytes does not suggest a major role for collagen-binding integrins in SRC.

The data we have presented show that SRC did not produce normal quantities of intact cell-surface anx V. This deficiency likely contributes to the inability of SRC cells to promote the chondrocyte-ECM binding to the extent essential for normal function of cartilage.

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