American Journal of Pathology, Vol. 142, No. 2, February 1993

Gap Junctional Communication between Vascular Cells

Induction of Connexin43 Messenger RNA in Macrophage Foam Cells of Atherosclerotic Lesions

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The structure and function of blood vessels depend on the ability of vascular cells to receive and transduce signals and to communicate with each other. One means by which vascular cells have been shown to communicate is via gap junctions, specifically connexin43. In atherosclerosis, the normal physical patterns of communication are disrupted by the subendothelial infiltration and accumulation of blood monocytes, which in turn can differentiate into resident foam cells. In this paper we report that neither freshly isolated human peripheral blood monocytes nor differentiated monocytes/macrophages exhibit functional gap junctional dye transfer in homo-cellular culture or in co-culture with endothelial cells or smooth muscle cells. By Northern analysis, neither freshly isolated blood monocytes nor pure cultures of differentiated monocyte/macrophages expressed gap junction messenger RNA. However, immunohistochemical staining followed by in situ hybridization on sections of human atherosclerotic carotid arteries revealed strong expression of gap junction connexin43 messenger RNA by macrophage foam cells. These results suggest that tissue-specific conditions present in atherosclerotic arteries induce expression of connexin43 messenger RNA in monocyte/macrophages. (Am J Pathol 1993, 142:593–606)

Gap junctions (Gjs) allow the direct exchange of cytoplasmic molecules between cells via aggregates of interconnecting channels. Each channel is comprised of a pair of hemichannels or "connexons," one each from apposing cells.[1,2] Each connexon is believed to be made of six identical polypeptide subunits termed "connexins" (Cxs).[3] There are strong sequence homologies[4,5] and a similar membrane topology[6,7] for several of the fully sequenced Cxs, ie, Cx43, -32, -26, -31, -40, -42, and -45. Different cell types are known to express different junctional proteins, and related but nonidentical Cxs appear to have a tissue-specific distribution.[4,8] The membrane topology of a typical Cx consists of a highly conserved amino terminus, four transmembrane domains, and two extracellular domains. In contrast, the cytoplasmic carboxyl terminus and the cytoplasmic loop are highly divergent in terms of length and sequence and may be responsible for differences in the properties of Gj channels of particular organs or tissues.[8]

The exchange of molecules between cells is nonspecific because of the large size of the intercellular channels formed by the connexon pairs (molecules up to 1 kd pass through). Ions and small metabolites in each cell, including nucleotides, sugars, amino acids, small peptides, and the second messengers Ca^{++}, inositol trisphosphate, and cyclic adenosine monophosphate have been shown to exchange via Gjs.[8] Physiological factors that regulate Gj intercellular communication (GJIC) include expression of the v-*src* oncogene,[9] intracellular Ca^{++} lev-

Supported by National Institutes of Health grant HL36049 from the National Heart, Lung, and Blood Institute. RL was partially supported by United States Public Health Service grant R37 HL21788 to M. F. Arnsdorf.

Accepted for publication July 27, 1992

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els,[2,8] platelet-derived growth factor,[8,10] phorbol esters,[11,12] retinoic acid,[8,13] extracellular adenosine triphosphate,[14] hormone stimulation via passage of second messenger molecules such as cyclic adenosine monophosphate,[8,15] low density lipoprotein,[16] cigarette smoke condensate,[16,17] tissue-specific extracellular matrix (ECM) components,[18] and intercellular adhesion molecules.[16–21] Inhibition or stimulation of GJIC controls the short-range homeostasis in cell groups or tissues. Dysfunction of this control mechanism in tumor promotion,[16,22] in embryonic development,[23] and in the functions of differentiated groups of cells in a tissue[15] suggests that GJIC is critically important in the control of cell proliferation and differentiation. GJIC can be regulated transcriptionally and/or post-transcriptionally at the levels of 1) message half-life, 2) protein synthesis, or 3) functional assembly of already synthesized Gj protein.[9] In addition, Gjs can be "gated," a modulation of open and closed states of assembled junctional channels, allowing for rapid control of GJIC.[7,24]

The presence and distribution of Gj structures in vascular endothelium have been documented in ultrastructural studies[25,26] and functional studies.[27,28] Vascular smooth muscle cell/smooth muscle cell (SMC/SMC) Gjs have been demonstrated by positive immunohistochemical staining for Cx43 protein and in dye transfer studies.[29] In addition, heterocellular junctions (endothelial cell (EC)/SMC; myoendothelial) have been observed.[30,31] Myoendothelial structures occur in arteries where cell processes extend through fenestrations in the internal elastic lamina and, more extensively, in arterioles and postcapillary venules. In culture, ECs, SMCs, and pericytes engage in homocellular as well as heterocellular junctional transfer, as evidenced by transfer of small molecules from an injected cell to its neighbors.[27,32–34] Recent studies have demonstrated that *in vitro* both ECs and SMCs express Cx43 exclusively,[32] suggesting a functional mechanism for metabolic/electrical integration of the vessel wall as well as a mechanism for the transduction of signals via second messenger molecules.

In atherogenesis, circulating monocytes enter the arterial wall, where they accumulate as lipid-filled macrophage foam cells. In this paper, we report that, although human peripheral blood monocytes or monocyte/macrophages, unlike ECs and SMCs, do not express messenger RNA (mRNA) for known Gj proteins or engage in GJIC *in vitro*, macrophage foam cells in human atherosclerotic lesions strongly express Cx43 mRNA.

Materials and Methods

Hybridization Probes

Both complementary DNA (cDNA) probes and riboprobes were generated from the following cDNAs that had been cloned into transcription vectors. The cDNA clone (G2A) of rat Cx43 mRNA, isolated and characterized as described previously,[2] corresponded to a 1393-nucleotide probe containing the amino terminus and coding region of Cx43 cDNA. The G2A insert was subcloned into the RNA transcription vector Bluescript M13+ (Stratagene, La Jolla, CA). Radiolabeled antisense and sense riboprobes were generated from this clone with T3 and T7 RNA polymerase (Stratagene), respectively, and uridine ^{35}S-triphosphate (1000 Ci/mmol; Amersham, Arlington Heights, IL). Uridine ^{35}S-triphosphate (300 μCi; 12 μmol/l final concentration) was used to label 1 μg of linearized plasmid DNA in the presence of 40 μmol/l unlabeled uridine triphosphate. Specific activities were in the range of 2 to 3 × 10^8 dpm/μg. Antisense transcripts were generated from the *Bam*HI-linearized plasmid and sense transcripts from the *Sal*I-linearized plasmid. Transcription reactions were performed according to the manufacturer's protocols. The cDNA of Cx32 mRNA was isolated and characterized by Paul[36] and corresponds to a 1494-nucleotide probe containing the amino terminus and complete coding region of Cx32 cDNA and approximately 613 base pairs of the 3′ untranslated region. This cDNA insert of Cx32 (clone 7A) was subcloned into the RNA transcription vector pGEM-3 (Promega, Madison, WI). Transcript sizes were confirmed by autoradiography of labeled probes on 1.5% agarose gels. Radioactive cDNA probes were generated by hexanucleotide priming by using deoxycytidine [^{35}P]triphosphate (3000 Ci/mmol; Amersham), according to the kit manufacter's directions (Amersham).

Tissue Sections

Specimens from human carotid endarterectomies were obtained at the time of surgery, washed briefly in phosphate-buffered saline (PBS), dissected, positioned correctly for sectioning, covered in OCT, and immediately immersed in isopentane and dry ice. Frozen specimens were stored at −80 C until sectioning. Frozen tissues were cut with a cryostat (−20 C) into 6- to 8-μm sections and were collected on gelatin/chromalum-coated slides. After standing at room temperature for 5 min, slides were dipped for 20 minutes at room temperature in freshly prepared 4% paraformaldehyde dissolved in PBS, followed by

sequential dehydration for 2 minutes each in 70%, 95%, and 100% ethanol. Slides were then air-dried and stored dessicated at −80 C.

Cell Isolation and Culture

Human umbilical vein endothelial cells (HUVEC) and smooth muscle cells (HUVSM) were isolated[28] and used at passages 1 to 5. HUVEC were plated onto dishes that had been coated with a 0.1% solution of gelatin, and the cells were maintained in M199 medium (GIBCO BRL, Grand Island, NY) supplemented with 20% fetal bovine serum, 1.0 mmol/l L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml heparin, and 50 μg/ml endothelial cell growth factor (Boehringer Mannheim Biochemicals, Indianapolis, IN). HUVSM were maintained in M199 medium supplemented with 20% fetal bovine serum, 1.0 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Human monocytes were obtained by a modification of the Recalde method.[37] In brief, anticoagulated blood was mixed with Plasmagel and allowed to settle into a bottom layer containing red cells and polymorphonuclear cells and a mononuclear leukocyte-rich upper layer. The upper layer was removed, washed, resuspended in citrated plasma, and made hypertonic with NaCl, causing the lymphocytes to become more dense while the monocytes resisted the density change. Monocytes were then separated from the heavier lymphocytes by density centrifugation on Ficoll-Paque, recovered from the interface, and plated in tissue culture plastic dishes in Dulbecco's modified Eagle's medium/20% autologous serum at 37 C for 30 minutes, after which time monocytes adhered and contaminating nonadherant lymphocytes were removed by a brief wash with PBS. For co-culture experiments, the monocytes (95 to 100% pure) were immediately removed from the tissue culture dishes by a brief washing in calcium- and magnesium-free PBS, followed by a 5-minute incubation in calcium- and magnesium-free PBS, 5.0 mmol/l ethylenediaminetetraacetic acid (EDTA), pH 7.2 at 37 C, after which all adherent monocytes had detached. Cells were then pelleted at low speed, resuspended in Dulbecco's modified Eagle's medium/20% autologous serum, and plated onto either HUVEC or HUVSM monolayers. Co-cultures were maintained in Dulbecco's modified Eagle's medium/20% autologous serum.

RNA Isolation and Northern Hybridization

Total RNA was isolated from tissue or from confluent monolayers of HUVEC and HUVSM by homogenization in guanidine thiocyanate and isopycnic centrifugation through cesium chloride.[38] Purified RNA was denatured with glyoxal, resolved by electrophoresis in 1.0% agarose, and then transferred by capillary blotting to a nylon support (ZetaProbe; Bio-Rad Laboratories, Richmond, CA) for hybridization with radiolabeled riboprobes. For hybridization with ^{32}P-labeled cDNA probes, blots were prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% bovine serum albumin, 1% glycine, 0.6% sodium dodecyl sulfate (SDS), 100 μg/ml herring sperm DNA, for 16 hours at 42 C in the same prehybridization solution with the exceptions that 1% glycine was omitted and 5% dextran sulfate and the labeled probe were added. The blots were washed three times in 2× standard saline citrate (SSC), 0.2% SDS, 1× Denhardt's solution, at room temperature for 15 minutes, followed by two washes in 0.1% SDS at 65 C for 20 minutes before autoradiography was performed at −70 C with an intensifying screen. For riboprobe hybridization, blots were prehybridized at 65 C for 1 to 2 hours and then hybridized overnight at the same temperature. Prehybridization and hybridization solutions were the same (50% formamide, 5× SSC, 50 mmol/l TRIS-HCl, pH 7.5, 0.1%, w/v, sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrolidone (M_r 40,000), 0.2% Ficoll (M_r 400,000), 0.5 mmol/l EDTA, 150 μg/ml denatured salmon sperm DNA), except for the addition of radiolabeled riboprobe (2.0×10^5 cpm/lane) to the hybridization solution. All washes were performed at 65 C, ie, two washes in 2× SSC/0.1% SDS for 15 minutes each, followed by two washes in 0.1× SSC/0.1% SDS for 15 minutes each. Blots were dried thoroughly and autoradiographed with Kodak X-OMAT AR film.

In Situ *Hybridization*

The protocol for *in situ* hybridization was based on that of Higgins and Wilson.[39] After tissue fixation, slides were stored at −80 C for up to 2 weeks. Slide boxes were allowed to warm to room temperature before opening. Slides were then removed and rehydrated in PBS for 5 minutes at room temperature, followed by immersion in 50 mmol/l TRIS-HCl (pH 7.6), 5 mmol/l EDTA, containing 50 μg/ml proteinase K, for 7.5 minutes at 37 C. This was followed by rinsing of the slides in PBS and immersion in 0.02 N HCl for 10 minutes at room temperature. After a final rinse in PBS, the slides were again fixed in 4% paraformaldehyde for 5 minutes and dehydrated in graded ethanol solutions containing 0.33 mol/l sodium acetate. Thirty microliters of prehybridization mixture containing 50% formamide, 0.3 mol/l NaCl,

10% dextran sulfate, 1× Denhardt's solution, 0.2% SDS, 40 mmol/l dithiothreitol, 250 μg/ml sheared herring sperm DNA, and 250 μg/ml yeast transfer RNA were applied to each dry tissue section and the slides were incubated for 2 to 3 hours at 45 C in a covered container humidified with 50% formamide in 2× SSC.

The hybridization mixture was exactly the same as the prehybridization mixture except for the addition of riboprobes. Antisense and sense riboprobes were diluted to the appropriate concentration in prehybridization mixture and added to the slides in a volume of 5 μl (final concentration of probe, 0.2 to 0.3 μg/ml hybridization solution). Slides were then incubated at 45 C overnight, after which they were removed from the incubator, allowed to drain briefly, immersed in 4× SSC containing 20 mmol/l 2-mercaptoethanol, and washed in several changes of 4× SSC. They were then treated with 20 μg/ml RNAse A in 0.5 mol/l NaCl, 10 mmol/l TRIS-HCl (pH 8.0), 1 mmol/l EDTA, for 30 minutes at 37 C and were rinsed in the same buffer for 30 minutes at 37 C, in 2× SSC for 30 minutes at room temperature, and in 0.1× SSC for 30 minutes at 45 C. After the posthybridization washes, slides were air dried and apposed to Kodak X-OMAT AR film for 48 hours to obtain x-ray images. They were then dipped in Kodak NTB-2 emulsion, exposed for 10–14 days, developed with D19 (Kodak, Rochester, NY), stained with Harris-modified hematoxylin (Fisher Diagnostics, Orangeburg, NY), mounted with Permount, and analyzed by using light microscopy (Technical Pan film, Kodak).

Specificity of the Cx43 riboprobe used for *in situ* hybridization was evaluated by Northern hybridization with total RNA isolated from rat heart. As a specificity control in the *in situ* hybridization assay, serial tissue sections were hybridized with ^{35}S-labeled riboprobes in the sense orientation under the same hybridization and washing conditions used for the antisense probes.

Intracellular Injection of Fluorescent Dyes

Lucifer Yellow (LY) dye was injected and GJIC was assayed as described previously.[7] Briefly, LY (M_r 457; Molecular Probes, OR) was dissolved in 0.15 mol/l lithium chloride buffered to pH 7.6 with 0.1 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, at a final concentration of 5.0% LY (w/v), and was filtered through a 0.22-μm nylon mesh (Centrex disposable microfilter units; Schleicher and Schuell, Keene, NH). For intracellular injections, microelectrodes were drawn from Oregon Dot borosilicate tubes (o.d., 1.2 mm; AM Systems, Mount Everett, WA). The electrodes were connected to electrometers containing active bridge circuits (Axoclamp 2A; Axon Instruments, Foster City, CA) and usually had a resistance of 25 to 50 MΩ when filled with 0.15 mol/l LiCl. For most studies, isolated pairs or groups of cells with distinct monolayer profile were selected. Dye was injected by passing a 3- to 10-nA hyperpolarizing (negative) current pulse at 1 Hz for 20 to 30 seconds or 5-nA negative direct currents for 2 to 5 minutes. Occasionally, dyes would diffuse passively (probably when an electrode tip diameter was large). Dye injection and spread were monitored on a fluorescence microscope (Nikon Diaphot, Nikon Instruments) with 100-W mercury arc lamp illumination, using epifluorescence optics with appropriate excitation and barrier filters (LY: excitation filter, 485 nm; barrier filter, 520 nm; plus an additonal KP 560-nm filter; rodamine: excitation filter, 540 nm; barrier filter, 590 nm). The extent of dye spread was recorded on ultrafast color film (ASA 3200; Konica USA Inc., Englewood Cliffs, NJ) 5 to 10 seconds and 3 to 5 minutes after injection.

Loading of Macrophages with Fluorescent Beads

In order to distinguish human macrophages from HUVEC or HUVSM in co-cultures, macrophages were labeled with red-fluorescing Covaspheres (Covaspheres CX, 1.0-μm diameter; Duke Scientific, Palo Alto, CA) before being plated onto monolayer cultures of HUVEC or HUVSM. Briefly, adherent macrophages were incubated with Covaspheres at a concentration of approximately 2.0×10^8 beads/ml, in a volume of 2.0 ml, in a 35-mm-diameter tissue culture dish at 37 C for 2 hours, during which time the cells internalized the beads. Free beads were then removed by washing of the macrophages twice with PBS, and finally the cells were released from the dish by incubation with calcium- and magnesium-free PBS, 5.0 mmol/l EDTA, pH 7.2, for 5 minutes at 37 C. Loaded cells were diluted to 10 ml with PBS and pelleted by low speed centrifugation. Pelleted cells were then immediately resuspended in Dulbecco's modified Eagle's medium/20% autologous serum, counted, and plated at a ratio of 1:2 (macrophages/HUVEC or HUVSM) onto subconfluent monolayer cultures on standard glass coverslips (25-mm diameter; Bellco Glass Inc., Vineland, NJ) placed in tissue culture wells. Bead-loaded macrophages were cocultured for at least 1 hour at 37 C before the coverslips were washed to remove unattached cells and were used in dye injection experiments. Bead-loaded macrophages were easily identifiable by their

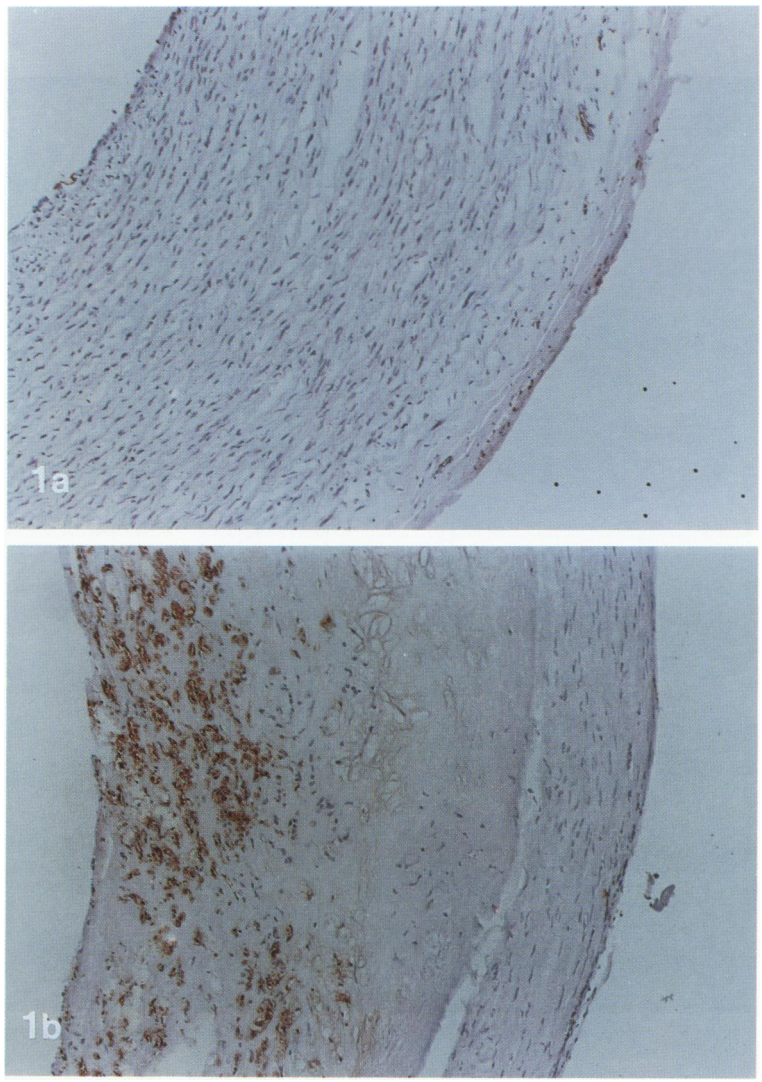

Figure 1. a: *Low power photomicrograph of normal human postmortem carotid artery stained with HAM56 Ab (×250).* b: *Atherosclerotic human carotid artery stained with HAM56 Ab (×250). Both sections were counterstained with hematoxylin.*

uptake of approximately three or four beads/cell, as visualized under a fluorescence microscope.

Immunocytochemisty

Slide boxes containing fixed cryosections were allowed to warm to room temperature before opening. Slides were rehydrated for 5 minutes in PBS at room temperature, followed by treatment with methanol/3.0% H_20_2 (4:1) in order to destroy endogenous peroxidase activity in the arterial tissue. Three washes (10 minutes each) with PBS/3% bovine serum albumin preceded incubation with the primary antibodies (Abs). HAM56[40] is characterized as a monoclonal mouse anti-human macrophage IgM Ab (1:20 dilution) recognizing an unspecified antigen (Enzo Diagnostics, Syosset, NY), and HHF35[41] is characterized as a monoclonal mouse antiactin Ab that recognizes actin isotypes common to all muscle cells (1:20 dilution) (Enzo Diagnostics). Slides were incubated with primary Abs for either 30 minutes at 37 C or overnight at 4 C, in a humidified chamber. After two washes in PBS (10 minutes each), secondary horseradish peroxidase-conjugated Abs (1:500 dilution) were reacted with the appropriate slides; horseradish peroxidase-conjugated affinity-purified goat anti-mouse IgM (Cappel, West Chester, PA) was reacted with HAM56 slides and horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Pierce, Rockford, IL) with HHF35 slides. Secondary Abs were incubated with slides for 30 minutes at room temperature. The slides were then washed three times (10 minutes each) in PBS and reacted for 5 to 7 minutes at room temperature with 0.02% H_2O_2/0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) (Pierce) prepared in 0.05 mol/l TRIS-HCl, pH 7.5.

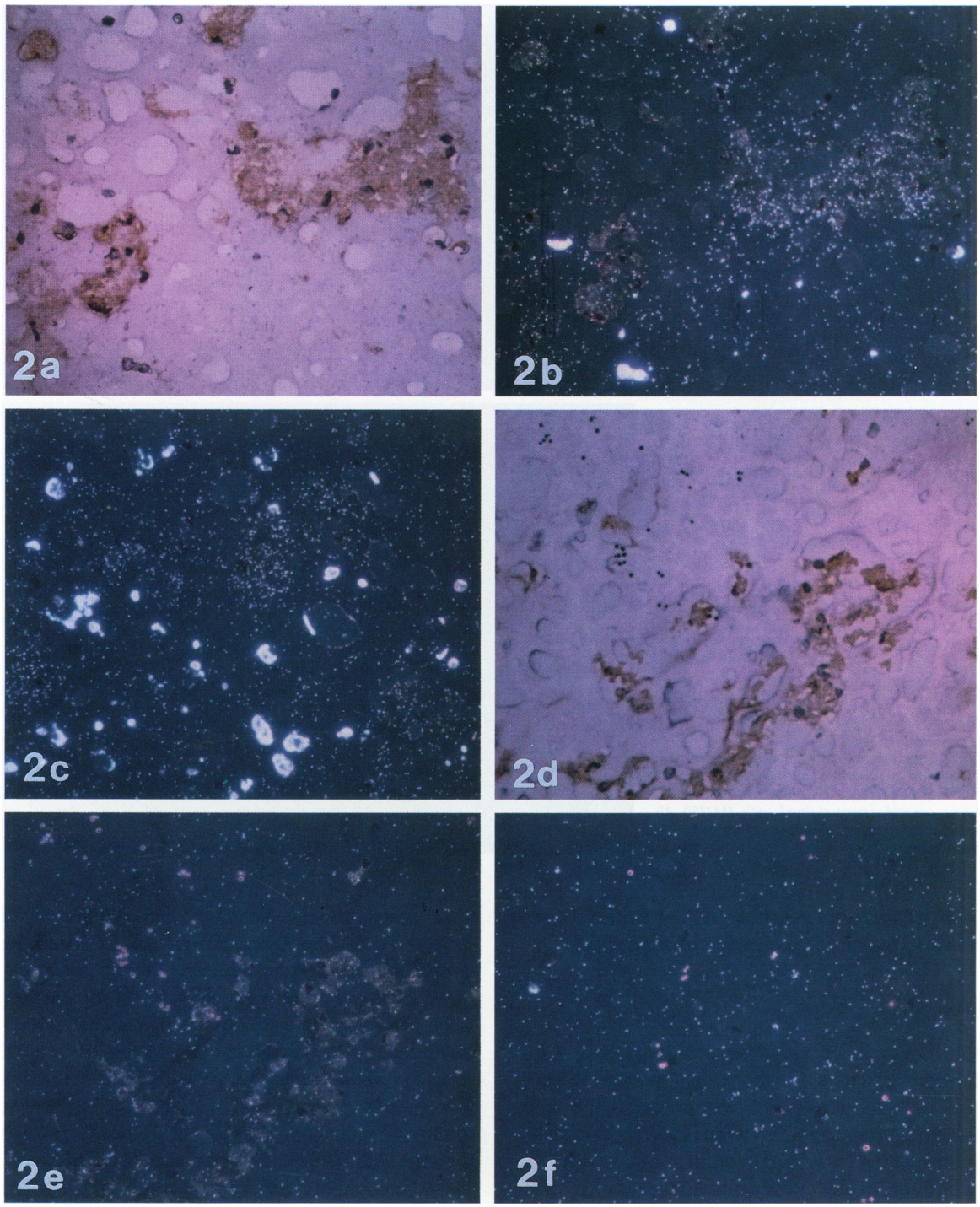

Figure 2. a: *Bright-field photomicrograph of atherosclerotic intima from a carotid artery stained with HAM56 Ab, hybridized with a ^{35}S-labeled Cx43 antisense riboprobe, and counterstained with hematoxylin.* b: *Dark-field photomicrograph of same field as in* a. c: *Dark-field photomicrograph of another area of the same lesion as in* b *but without prior immunostaining with HAM56 Ab.* d: *Bright-field photomicrograph of another area from the same lesion, stained with HAM56 Ab, hybridized with an ^{35}S-labeled Cx43 sense riboprobe, and counterstained with hematoxylin.* e: *Dark-field photomicrograph of same field as in* d. f: *Dark-field photomicrograph of another area of the same lesion as in* d *but without prior immunostaining with HAM56 Ab* (a-f, ×400).

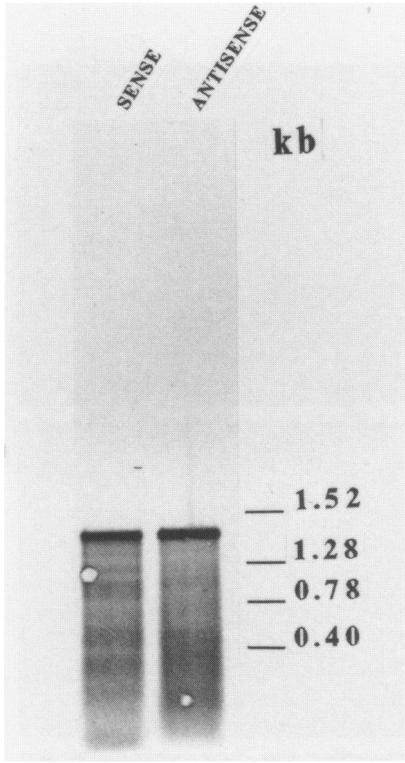

Figure 3. *Audioradiogram of Northern blot of ^{35}S-labeled sense and antisense Cx43 riboprobes used in the in situ hybridization experiments. Note that both labeled riboprobes are predominantly in the form of a full length transcript generated from the Cx43 1.4-kb cDNA.*

After staining with DAB, slides were washed twice (3 min) in distilled water at room temperature and either counterstained with hematoxylin followed by dehyration and mounting or prepared for *in situ* hybridization beginning with the proteinase K treatment.

Results

Cx43 Expression in Macrophage Foam Cells of Atherosclerotic Carotid Artery

Cryostat sections (6- to 8-μm thickness) of normal artery obtained at postmortem and atherosclerotic carotid artery tissue removed during endarterectomy surgery were stained immunohistochemically to the identify cell types present. As shown in Figure 1b, regions of macrophage foam cell accumulation were clearly distinguished from adjacent SMCs and were associated with dense ECM. By way of comparison (Figure 1a), normal postmortem carotid artery showed ordered layers of medial SMCs with no HAM56 Ab staining. Figure 2 shows combined immunohistochemical and *in situ* hybridization of cryostat sections from a representative human carotid endarterectomy specimen. The tissue consisted predominantly of intima, containing mostly macrophages. Immunohistochemical staining with the macrophage-specific HAM56 Ab revealed the presence of numerous aggregated macrophage foam cells surrounded by ECM material (Figure 2a). Subsequent *in situ* hybridization of the same section with a ^{35}S-labeled antisense Cx43 riboprobe resulted in preferential labeling of macrophages, observable as localized increased grain densities in the dark-field photomicrograph (Figure 2b). Adjacent sections from the same tissue when hybridized with sense Cx43 riboprobe (Figure 2, d and e) exhibited low random labeling at a grain density similar to the background observed in the antisense hybridization.

Because it was essential to first immunochemically stain and then probe the same tissue section by *in situ* hybridization, potential artifacts of this double procedure were investigated. First, because our secondary Ab detection system depended upon the horseradish peroxidase-linked reaction, tissue sections were pretreated with H_2O_2 in order to destroy macrophage endogenous peroxidase activity. Comparison of untreated and pretreated tissue sections in reaction with the DAB complex alone revealed some faint staining of the untreated specimens, which was completely abolished by pretreatment with H_2O_2. Second, nonspecific binding of the riboprobe to the DAB complex resulting from the immu-

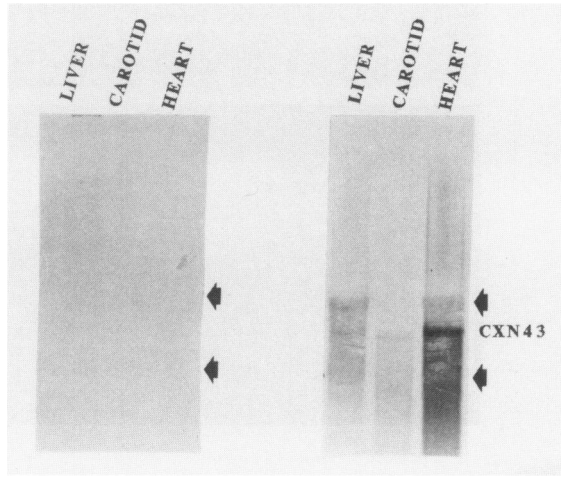

Figure 4. **Left,** *autoradiogram of Northern blot of total cell RNA (10 μg of RNA/lane) from rat liver, human atherosclerotic carotid artery, and rat heart tissue, hybridized with the ^{35}S-labeled sense Cx43 riboprobe used in the* **in situ** *hybridization experiments.* **Right,** *autoradiogram of similar Northern blot as at left but hybridized with the ^{35}S-labeled antisense Cx43 riboprobe used in the in situ hybridization experiments. Note the prominent 3.0-kb signal corresponding to rat heart Cx43 mRNA. Human atherosclerotic carotid artery RNA contains a similar band at a position slightly lower than the 3.0-kb position. Arrows, positions of 28 S and 18 S ribosomal RNAs.*

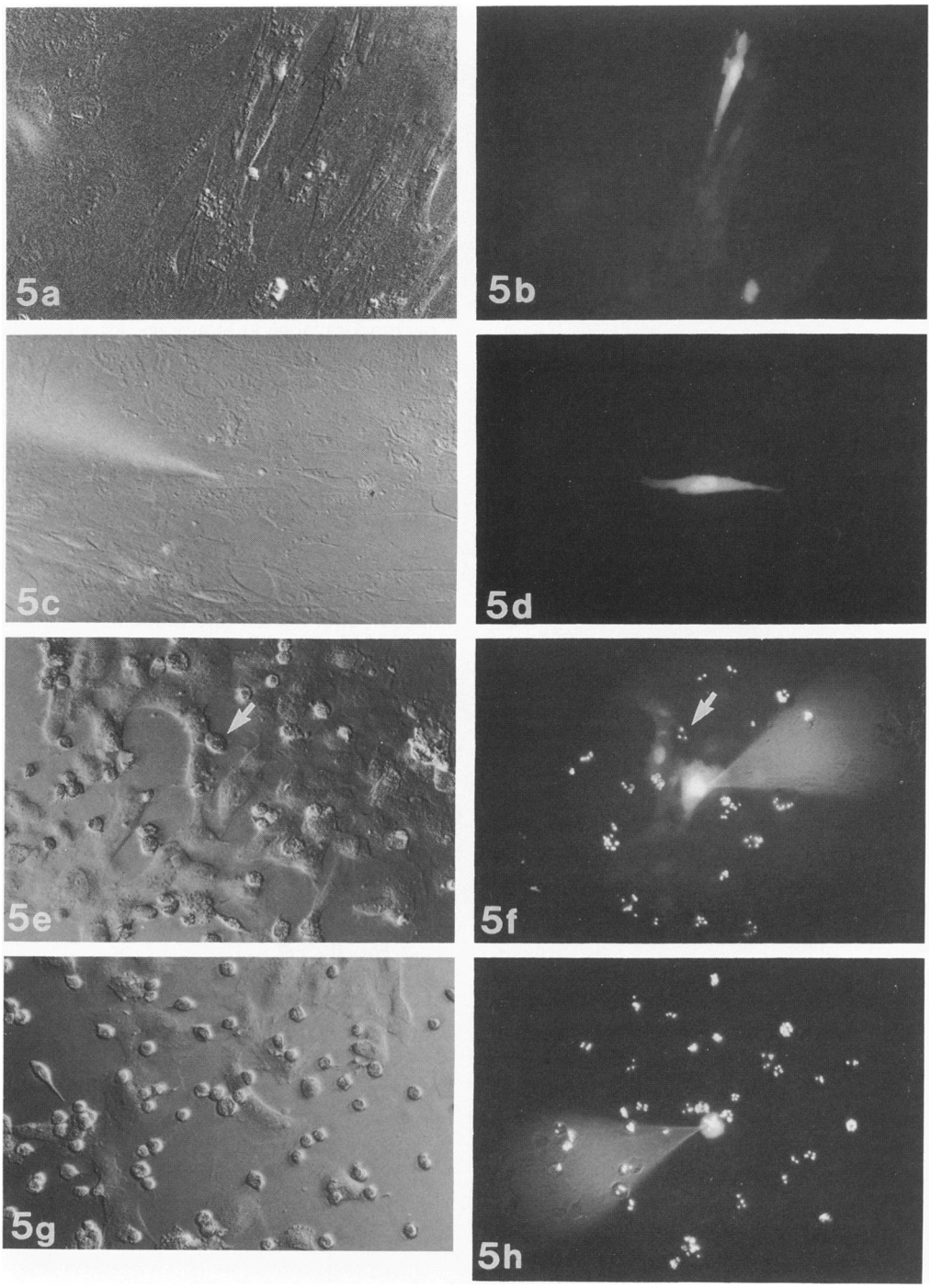

Figure 5. *Phase-contrast* (a, c, e, *and* g) *and fluorescence micrographs* (b, d, f, and h) *of dye injections (LY) into single cells of homocellular and hererocellular cultures in vitro.* a *and* b: *Dye injected into a single cell of a homocellular culture of HUVSM* (a) *transfered via Gj to neighboring cells, as seen by the spread of fluorescence in* b. c *and* d: *Dye injected into a single cell after preincubation of homocellular HUVSM culture with phorbol myristate acetate (1×10^{-7} mol/l), an agent known to inhibit GJIC, is shown. Note lack of detectable transfer to adjacent cells in* d *and the presence of fluorescent microelectrode in* a. e *and* f: *Dye injected into a single HUVEC passed to adjacent HUVEC but not to adjacent monocyte/macrophages in HUVEV/macrophage co-culture* (f). *Note that the monocyte/macrophages were prelabeled with fluorescent marker beads (small white beads in photographs) in order to dinstinguish them from HUVEC in co-culture. Failure of transfer is particularly evident in the macrophage marked with the white arrow.* g *and* h: *Dye injected into a single human monocyte/macrophage failed to spread to either other macrophages or HUVEC* (h). *The paired photomicrographs in* a *and* b, c *and* d, e *and* f, *and* g *and* h *are of the same field (×400).*

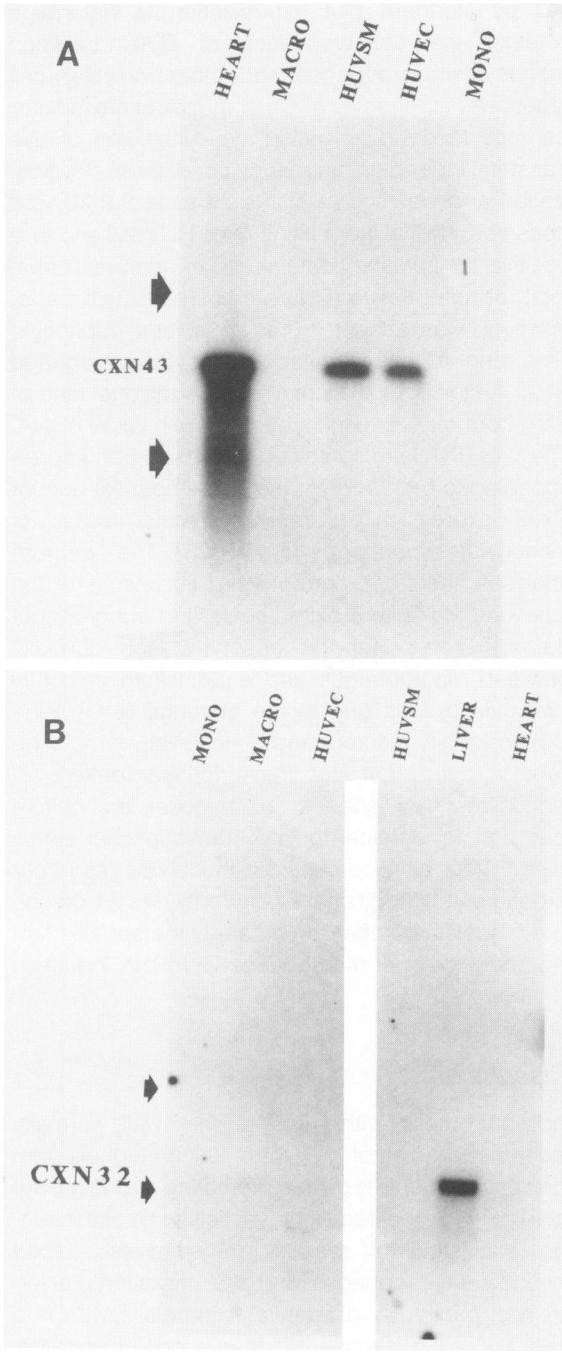

Figure 6. A: *Audioradiogram of Northern blot of total cell RNA (10 μg of RNA/lane) hybridized with a ^{32}P-labeled Cx43 cDNA probe. RNA samples were from rat heart tissue, human monocyte-derived macrophages (5 days in tissue culture; see Materials and Methods) (MACRO), cultured HUVSM, cultured HUVEC, and freshly isolated human blood monocytes (MONO) that had not adhered to any surface. Rat heart RNA served as the positive Cx43 control.* B: *Audioradiogram of Northern blot as in* A *except for the inclusion of rat liver Cx32 positive control. Blot* B *was hybridized with a ^{32}P-labeled Cx32 cDNA probe. Arrows, position of the 28 S and 18 S ribosomal RNAs.*

nohistochemical reaction appeared unlikely, because in Figure 2e there was no preferential binding of the sense riboprobe to the DAB reaction product. However, because this background was observed with the sense probe (which appears to have a lower nonspecific background binding to total cell RNA, and potentially other cell constituents as well) (see Figure 4) we performed an additional check for any possible interaction of the DAB complex with the antisense radioactive probe by hybridizing sections that had not been previously immunostained. As shown in Figure 2c, the pattern of specific (localized over dark blue cell nuclei) and nonspecific hybridization of the antisense riboprobe was identical to that in Figure 2b, where the macrophages had been immunostained. It should also be noted that the background signal of the sense riboprobe was similar whether the macrophages were immunostained (Figure 2e) or unstained (Figure 2f). Furthermore, the integrity of the riboprobes was evaluated by 1.0% agarose gel electrophoresis (Figure 3), which revealed one major 1400-nucleotide (full length) band for both sense and antisense transcription products.

As shown in Figure 4, the antisense riboprobe specifically hybridized to the 3.0-kilobase (kb) rat Cx43 mRNA and to an RNA species in human atherosclerotic carotid artery tissue in the same region. The antisense riboprobe also weakly hybridized to the 28 S and 18 S ribosomal RNA species, whereas the sense probe exhibited no nonspecific binding. The low specificity binding of the antisense probe would most likely be removed during the *in situ* protocol by the RNAse digestion step. In an attempt to mimic the *in situ* protocol, we incubated the hybridized blot with RNase under conditions identical to the *in situ* protocol and found that this step completely removed the weak binding of the antisense probe to the ribosomal RNA on the Northern blot without affecting the specific binding to the 3.0-kb Cx43 band (data not shown).

Absence in Monocyte/Macrophages in Vitro *of Functional GJIC Present in ECs and SMCs*

Near-confluent homocellular monolayers (3 to 5 days in culture) of HUVEC and HUVSM were tested for their ability to carry out Gj-mediated transfer of the fluorescent dye LY. Single cells were injected, after which the electrode was removed and GJIC was determined by the spread of dye to neighboring cells. Dye transfer to neighboring cells was detected within seconds after injection (Fig. 5, a and b). Preincubation of HUVSM with phorbol myristate acetate, a known inhibitor of GJIC, for 30 minutes blocked dye transfer by closing Gj channels (Figure 5, c and d).

GJIC between vascular cells and monocyte/macrophages was examined in co-cultures of HUVEC/macrophages and HUVSM/macrophages. Subconfluent monolayers of HUVEC (2 to 3 days in culture) were overlaid with pure populations of human blood monocyte/macrophages that had been maintained in culture for 2 to 3 days. In order to distinguish the macrophages in the co-culture, they were preloaded with red fluorescent Covaspheres via phagocytosis. Cells were co-cultured for at least 1 hour, followed by two washes with complete medium to remove nonadherent macrophages. In time-lapse video recordings of macrophage/endothelial co-cultures, we have noted that, after an extended period of macrophage adhesion and spreading onto a confluent HUVEC monolayer, macrophages moved beneath the monolayer where they continued complex migratory patterns. Consequently, dye transfer studies were restricted to the period of attachment and spreading on subconfluent endothelial monolayers. In a series of heterologous cell pairs, either a HUVEC or a macrophage was injected with LY. As shown in Figure 5f, dye injected into a single HUVEC spread rapidly to adjacent ECs but was excluded from adherent macrophages in the co-culture. In a complementary experiment, macrophages were injected with LY. The dye was retained in the injected cell with no evidence of transfer to adjacent ECs (Figure 5, g and h), demonstrating the absence of functional Gjs between ECs and macrophages under our co-culture conditions. Other macrophages in contact with the injected macrophage also failed to take up the dye, demonstrating that macrophages did not form functional Gjs with each other. Homocellular cultures of unlabeled macrophages also failed to transfer dye (data not shown). In co-culture, labeled macrophages with red fluorescent beads were distinguished unambiguously from macrophages containing LY by the use of appropriate filters. Thus, *in vitro* we found no functional evidence suggesting monocyte/macrophage GJIC with endothelium. However, manipulations of co-culture conditons such as duration of monocyte/macrophage interaction with endothelium and activation of either or both cell types in co-culture may be required for the development of GJIC involving monocyte/macrophages. Purified macrophages failed to show significant binding to HUVSM in co-culture, a phenomenon that has been noted by others.[42]

Vascular Cell Gj mRNA Expression in Vitro

RNAs isolated from ECs, SMCs, and monocyte/macrophages were probed with cDNAs for Cx32 and -43 by Northern blot hybridization to determine whether the observed lack of GJIC between monocytes/macrophages and other vascular cell types was at the level of Cx43 mRNA expression or perhaps involved a "gating"-type inhibition of dye transfer. Under high stringency conditions of hybridization and washing, a single band at 3.0 kb was observed in RNA from HUVEC or HUVSM and in a positive control lane containing Cx43-rich RNA from total rat heart tissue (Figure 6A). The 3.0-kb band, however, was absent in freshly isolated monocyte RNA and in differentiated monocyte/macrophage RNA. A similar blot containing an additional lane of RNA from rat liver was hybridized with Cx32 cDNA. The liver RNA lane contained the 1.6-kb band corresponding to Cx32 mRNA; there was no evidence for Cx32 expression in heart, vascular cells, or monocyte/macrophages (Figure 6B). The Northern analyses, therefore, corroborated the results of the functional co-culture experiments and suggest, but do not prove, that the observed lack of GJIC between macrophages and endothelium in our *in vitro* system was due to the absence of Gj gene expression in macrophages. However, it must be kept in mind that the transcriptional pattern of monocytes/macrophages in homocellular culture may not be identical to their transcriptional status even 1 hour after co-culture with HUVEC (as in our experiments). Additional *in situ* analyses of co-culture models are necessary to study the specific timing and context of macrophage Gj mRNA induction *in vitro*.

Discussion

Individual cells within the arterial wall normally receive two types of growth-regulating signals from their environment, 1) growth promoting and 2) growth inhibiting. In atherosclerotic vessels, intimal thickening consisting of recently transmigrated blood monocyte/macrophages and the migration/proliferation of previously differentiated medial SMCs is a hallmark of the disease. Whether a cell proliferates, migrates, or differentiates into a postmitotic state depends upon the overall combination of signals it is receiving, the intercellular connections it makes with surrounding cells, and the contacts it makes with the specific ECM in which it resides. All of these factors have been shown to influence GJIC.[10,15,18–21] Recent reports in the literature have documented various chemotactic[43–46] and growth-promoting[43,44,47,48] signals present in atherosclerotic lesions that may participate in the *in vivo* accumulation, proliferation, and differentiation of

macrophage foam cells. In this paper, we have examined intimal macrophages of atherosclerotic lesions in relation to GJIC, a cellular mechanism associated with growth control and differentiation.

When human carotid endarterectomy tissues were analyzed by combined immunohistochemistry and *in situ* hybridization on the same cryostat section, the presence of mRNA homologous to Cx43 was detected within intimal macrophage foam cells. However, when blood-derived human monocyte/macrophages were cultured alone or co-cultured for 1 hour with either HUVEC or HUVSM, they did not engage in GJIC. Homocellular cultures of either HUVEC or HUVSM did exhibit GJIC. When Northern blots of total RNA from freshly isolated human monocytes, differentiated human monocyte-derived macrophages, HUVEC, and HUVSM were hybridized with cDNA probes for Cx32 or -43, HUVSM and HUVEC expressed a single 3.0-kb RNA homologous to Cx43, whereas monocytes or monocyte/macrophages did not. None of the vascular cells, monocytes, or monocyte/macrophages expressed RNA homologous to Cx32. These results suggest that tissue-specific factors present in the vessel wall in some way induce Cx43 gene expression in blood-derived macrophages of atherosclerotic lesions.

Regulation of Gj gene expression among vascular cells *in vitro* has been demonstrated by Navab et al.[49] Utilizing a vascular wall model consisting of a continuous and confluent EC monolayer on top of a thin layer of ECM over confluent layers of SMCs (with frequent direct contacts between the ECs and underlying SMCs), these authors showed that 1) co-culture of human arterial ECs and human arterial SMCs (ie., co-culture) resulted in levels of Cx43 mRNA that were 2.7-fold higher than could be accounted for by the sum of the contributions of the components (ie., monolayer cultures of either cell type), 2) a 2-hour exposure of human monocytes to confluent human arterial ECs (plated onto a layer of collagen) produced levels of total Cx43 mRNA that were 3.7-fold higher than could be accounted for by the contribution of monocytes (which contained no Cx43 mRNA in homocellular culture) and human arterial ECs cultured separately, and 3) monocytes plated onto co-cultures of human arterial SMCs/collagen/humanarterial ECs resulted in a marked increase in the induction of Cx43 mRNA to levels 2.8-fold greater than the combined values for culture of monocytes and co-culture of ECs and SMCs. These authors did not test functional GJIC, nor did they determine which cell types in the co-culture were responsible for the increase in total Cx43 mRNA. These data suggest that normal heterotypic interactions of vascular cells (ECs/SMCs) result in a general up-regulation of Cx43 gene expression in the vessel wall and, more pertinent to this manuscript, that the interaction of infiltrating monocytes with aortic tissue further stimulates Gj mRNA expression. In view of the fact that human monocytes cultured alone do not make Cx43 mRNA, our data are consistent with and extend the *in vitro* studies of Navab et al to the arterial wall. Our *in situ* results demonstrate that, under conditions of mononuclear leukocyte recruitment and foam cell formation in atherogenesis, the heterotypic cell interactions and perhaps interaction of migrating leukocytes with specific cytokines and/or ECM components induce macrophage expression of Gj mRNA.

In support of our finding that macrophages are capable of expressing Cx43 mRNA in response to an appropriate stimulus is the work of Beyer and Steinberg,[14] who have recently identified Cx43 homology with a pore in the plasma membrane of mouse peritoneal macrophages and of certain lines of J774 macrophages that is opened by extracellular adenosine triphosphate. Under conditions of high stringency, RNA isolated from J774 cells hybridized with cDNA for Cx43 but not with cDNA for Cxs32, -26, or -46. J774 lines that did not permeabilize in response to extracellular adenosine triphosphate did not hybridize with Cx43 cDNA.

Adherence of blood monocytes to the endothelium at sites of predilection for lesion development is the earliest observable cellular event in atherogenesis. There is extensive *in vitro* evidence[49–52] suggesting that high levels of native low density lipoprotein or low levels of mildly oxidized low density lipoprotein play a role in the induction of specific adhesion proteins on endothelium and, *in vivo*, a VCAM-1-like adhesion protein that supports the adhesion of blood mononuclear cells, but not polymorphonuclear cells, has been immunohistochemically identified by Cybulsky and Gimbrone[53] on atherosclerotic rabbit endothelium. After monocyte adhesion to "dysfunctional" endothelium, the monocyte migrates into the subendothelial space, most likely in response to one or more chemotactic stimuli such as platelet-derived growth factor[43,44] or monocyte chemoattractant protein-1,[45,49,52] and there undergoes lipid accumulation and proliferation/differentiation that ultimately results in the foam cell aggregates characteristic of atherosclerotic lesions. It will be important to ascertain at what point in this scenario macrophage Cx43 gene expression is stimulated and what role macrophage GJIC may play at the different stages of monocyte-to-foam cell differentiation and lesion development.

In the field of Gj biology there is mounting evidence that an intercellular adhesion event precedes and may be required for the establishment of functional GJIC between cells.[18–21] In this context, the demonstration of VCAM-1 expression by cytokine-activated human ECs and by certain tissue macrophages,[54] including a variable proportion of those found in lymph nodes, spleen, thymus, and Kupffer cells, supports the view that tissue-specific conditions present in atherosclerosis may promote the expression of this adhesion protein on macrophages of atherosclerotic lesions. VLA-4, the counter-receptor for VCAM-1, is present only on mononuclear leukocytes. If lesion macrophages do express VCAM-1, then VCAM-1/VLA-4-mediated aggregates of accumulating monocyte/macrophages may form and, in turn, lead to the induction of GJIC among these cells. Gjs are in general related to the control of cell proliferation and might be viewed as growth inhibitory, counteracting the reported mitotic activity of macrophages and macrophage foam cells in lesions.[55,56]

It will now be important to dissect the physiological (pathophysiological) conditions leading to monocyte/macrophage expression of Cx43 by using a combination of *in vitro* vascular cell wall models and *in situ* techniques applied both to the co-culture models and to additional *in vivo* models of macrophage accumulation including granulomas and xanthomas. Such studies may indicate how factors such as the local cytokine environment, lipid accumulation, and altered ECM may affect the expression of monocyte/macrophage Gj mRNA *in vitro* and in the vascular wall.

Acknowledgments

The authors thank Christopher Zarins, MD, for supplying normal and atherosclerotic human tissues, David Paul of Harvard Medical School for gifts of cDNAs for Cx32 and Cx43, John Lukens, Patrick Chang, and Kelly Gale for technical assistance, and René Payne for help in preparation of the manuscript.

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