Gap Junction Messenger RNA Expression by Vascular Wall Cells

David M. Larson, Christian C. Haudenschild, and Eric C. Beyer

Gap junctions between vessel wall cells provide a pathway for the intercellular exchange of ions and small molecules. Pure cultures of microvascular and macrovascular endothelial and smooth muscle cells, vascular pericytes, and several nonvascular cell lines were tested for junctional communication by fluorescent dye transfer. All of the vascular wall cells were capable of dye transfer. Since gap junctions are formed by a family of related proteins (connexins) whose unique domains may confer physiological regulatory properties, we tested total RNA from these cultures by Northern blot analysis for expression of the currently available, characterized, and cloned mammalian gap junction proteins: connexin26, connexin32, and connexin43. All of the vascular wall cells expressed connexin43 messenger RNA. Connexin43 was expressed in vascular cells from bovine, porcine, rat, and human sources. Several nonvascular cell lines of mesenchymal origin also expressed connexin43 messenger RNA. When high stringency Northern blots were used, messenger RNAs for connexin32 or connexin26 were not detected in any of the vascular wall cells but were expressed in several cell lines of epithelial origin. Freshly isolated and purified aortic endothelial and smooth muscle RNA preparations similarly contained only connexin43 messenger RNA, excluding the possibility of culture-induced alterations in gene expression. The expression of connexin43 by all vascular wall cells may provide a mechanism for the functional integration of the vessel wall by gap junctions. (Circulation Research 1990;66:1074–1080)

Gap junctions are membrane structures that contain aqueous channels linking the cytoplasm of adjacent cells.1,2 These structures are responsible for electrotonic coupling, equilibration of ionic and small molecular pools between coupled cells, and coordination of tissue responses to stimuli. Electron microscopic studies have demonstrated gap junctions between vascular wall cells throughout the vascular tree.2,3 We2–4 and others5 have previously shown evidence of junctional transfer of small molecules in the vessel wall in situ. Therefore, gap junctions exist between cells in the vessel wall and appear to be functional.

In culture, endothelial cells, smooth muscle cells, and pericytes all produce gap junctions and engage in junctional transfer among themselves.2,6–8 In addition, these cells are capable of producing functional gap junctions between cell types.2–5,9 In the vessel wall, heterocellular communication has also been detected.3–5

Recent biochemical and molecular studies have demonstrated that there is a family of related gap junction proteins called connexins. Well-characterized mammalian connexins include connexin43 (the “heart” gap junction protein)10–13 and connexin32 and connexin26 (“liver” proteins).14–17 In addition, complementary DNAs for connexin26, connexin32, and connexin43 have been cloned.12,15,17 Each of the proteins is expressed in a wide variety of tissues. The transmembrane and extracellular domains of these connexins are similar, suggesting that they form similar gap junction structures. However, they each also have unique cytoplasmic domains that may confer physiological specificity in different cells. It is not known which type(s) of gap junction proteins comprises the specific vascular junctions and whether they vary with vascular cell type, vascular bed, or species.

Therefore, the goals of the current study were to answer the following questions: Are there cell, vessel, or species differences in the capability of cultured vascular wall cells to engage in junctional transfer? Which connexin(s) is expressed by vessel wall cells in vivo and in vitro? Do endothelial cells, smooth mus-
ele cells, and pericytes all express the same connexin(s)? Are there species differences in expression?

**Materials and Methods**

**Cell Cultures**

Vascular wall cell cultures, unless otherwise noted, were grown in Dulbecco’s Modified Eagle Medium (DMEM, 100 mg% glucose; GIBCO Laboratories, Grand Island, New York) plus 10% calf serum (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO Laboratories).

**Endothelium.** Bovine and porcine aortic endothelial cultures were established from collagenase-digested razor-blade intimal scrapings by using a previously described technique.18 Bovine brain microvascular endothelial cells were isolated from calf cerebral cortex as described by Carson and Haudenschild19 and cultured on gelatin-coated dishes in DMEM plus 15% equine plasma-derived serum (Hyclone Laboratories), 20% bovine aortic endothelial cell–conditioned medium, 2% retinal extract, and 500 μg/ml heparin (Sigma Chemical, St. Louis, Missouri).6,19 Human corpus cavernosum endothelial cultures were established from surgical specimens20 and cultured in the same medium as the bovine brain microvascular endothelial cells. Human umbilical vein endothelial cells were supplied by W.J. Atkinson and M.A. Gimbrone, Jr., Brigham and Women’s Hospital, Boston, Massachusetts, and were grown on gelatin-coated dishes in M199 (M.A. Bio-products, Walkersville, Maryland) plus 20% fetal calf serum (FCS; Hyclone Laboratories), 50 μg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton, Massachusetts), and 100 μg/ml heparin (Sigma Chemical).21

**Pericytes.** Bovine brain and retinal microvascular pericytes were cultured as described previously.19 Human mesangial cells were a gift from R.J. Quig, Medical College of Virginia, Richmond. These cultures were established during isolation of glomerular epithelial cells from cadaveric kidney cortex by a combined mechanical and enzymatic dissociation protocol.22,23 After 10–12 days in DMEM+10% FCS, homogeneous mesangial cell colonies were collected by use of cloning rings and were cultured in the above medium. Mesangial cells were differentiated from epithelial cells on the basis of morphology and lack of staining with mouse monoclonal anti-human cytotactin immunoglobulin M (Enzo Biochemical, New York, New York). No further characterization was carried out.

**Vascular smooth muscle.** Bovine and porcine aortic smooth muscle cultures were established by explant outgrowth.24 Rat aortic smooth muscle cultures were established by enzymatic dissociation, as were cultures from rat mesenteric arterial arcades.25,26

**Nonvascular cells.** The following cell lines were acquired from American Type Culture Collection, Rockville, Maryland: mouse L929 fibroblasts (ATCC CCL1), rat C6 glioma cells (ATCC CCL107), rat AR42J pancreatic exocrine tumor cells (ATCC CRL1492), canine MDCK kidney epithelial cells (ATCC CCL34), mouse sarcoma 180 cells (ATCC TIB66), and human diploid lung IMR-90 fibroblasts (ATCC CCL186). MDCK and AR42J cells were grown in DMEM with 10% FCS. In addition, human colonic carcinoma T84 cells27 were the generous gift of C.F. Delp and J.L. Madara, Brigham and Women’s Hospital, Boston, Massachusetts, and were grown in DMEM/Ham’s F12 (1:1) with 6% newborn calf serum and antibiotics.

**Tissue Preparations**

Tissues were used to check on the in vivo expression of junctional messenger RNA (mRNA), where possible (see References 12, 15, and 16). Bovine or rat heart tissue RNA samples were used as positive controls for connexin43. Rat liver RNA samples were a positive control for connexin26 and connexin32.

The prime criterion for the studies on the vessel wall was collection of an essentially pure cell type. Living “Häutchen” preparations from bovine aortas were razor-blade intimal scrapings. This is the initial step in the usual culture procedure for bovine aortic endothelial cells and yields a nearly pure population of endothelium. Rat aortic medial preparations, cleaned of adventitia by dissection and of intima by scraping, were used as a pure source of smooth muscle cells.

**Dye Transfer Assays**

We have previously demonstrated junctional transfer in several of the cell types tested in this study (bovine aortic endothelial cells,7,18 bovine brain microvascular endothelial cells,6 bovine brain microvascular pericytes,6 rat aortic smooth muscle cells,2 and bovine aortic smooth muscle cells5,7). Parallel cultures of most of the cells used for RNA preparations in this study were tested by using either direct microinjection of Lucifer yellow CH (Sigma; for techniques, see References 2, 6, 7, 9, and 18) or the scrape-load dye transfer assay.18,28 The basis of the latter assay is that cells along the edge of a scrape are briefly permeabilized in a dye solution, trapping the dyes (Lucifer yellow CH and rhodamine-dextran) when they reseal their membranes. Rhodamine-dextran (70S, Sigma Chemical) is too large to diffuse through junctions and, thus, serves as an indicator of the originally loaded cells. The Lucifer yellow CH is junction permeant2–4,6,7,18,29 and its diffusion from cell to cell serves as an indicator of patent gap junctions.

**Complementary DNA Probes**

The cloning of DNA encoding for connexin32,15 connexin26,17 and connexin4312 has been previously described. The complementary DNA probes used in this study consisted of DNA fragments containing essentially only coding sequences prepared by digestion with appropriate restriction enzymes and isolation by electrophoresis in low-melting-point agarose.12,15,17 Probes were labeled with 32P by use of the Klenow fragment of DNA polymerase I and random hexanucleotide primers.30
TABLE 1. Cultures, Dye Transfer, and Northern Blots

<table>
<thead>
<tr>
<th>Culture</th>
<th>Species</th>
<th>Origin</th>
<th>Passage level</th>
<th>Dye transfer</th>
<th>Northern blots (Connexin43)</th>
<th>Northern blots (Connexin32)</th>
<th>Northern blots (Connexin26)</th>
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<tbody>
<tr>
<td>Vascular</td>
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<tr>
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<td>Bovine</td>
<td>Aortic</td>
<td>0–4</td>
<td>+ s,i</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>Brain microvessels</td>
<td>1,7</td>
<td>+ i</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>Porcine</td>
<td>Aortic</td>
<td>3</td>
<td>+ s</td>
<td>+</td>
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<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>Corpus cavernosum</td>
<td>3,4</td>
<td>+ i</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>Brain microvessels</td>
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<td>+ i</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>+ i</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>HMes</td>
<td>Human</td>
<td>Glomerular</td>
<td>4</td>
<td>+ s</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PASMC</td>
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<td>+</td>
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<td>–</td>
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<td>+ s,i</td>
<td>+</td>
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<tr>
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<td>Fibroblast</td>
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<td>–</td>
<td>+</td>
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<td>Sarcoma</td>
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<td>– s,i</td>
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<td>Rat</td>
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<td>46</td>
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<td>+</td>
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<td>–</td>
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<td>Rat</td>
<td>Exocrine pancreatic</td>
<td>NK</td>
<td>+ i</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>Kidney epithelium</td>
<td>NK</td>
<td>+/-~</td>
<td>–</td>
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<td>ND</td>
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<tr>
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<td>– s,i</td>
<td>–</td>
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<tr>
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<td>Lung fibroblast</td>
<td>12</td>
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<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

Passage levels tested (blots) were as follows: 0, primary culture; 1, first passage; . . . 589, passage 589. Dye transfer techniques included scrape-loading (s) and dye injection (i). +, Positive; –, negative; ND, not done; NK, not known.

*Data obtained from Reference 9.
†Data obtained from References 33 and 34.

Northern Blots

Total RNA was harvested from confluent cultures (no earlier than 3 days after passage) and fresh tissue samples by extraction and homogenization in 4 M guanidine isothiocyanate (enzyme grade, Bethesda Research Laboratories, Gaithersburg, Maryland), 25 mM sodium acetate (molecular biology grade, Sigma Chemical), 0.83% β-mercaptoethanol (Sigma Chemical), and 0.5% N-lauroylsarcosine (Sigma Chemical) (pH 6, sterile filtered). Guanidine isothiocyanate samples were centrifuged through a CsCl cushion to isolate RNA. RNA samples (10 μg/lane) were electrophoresed on 1% agarose/formaldehyde gels and capillary-blotted onto nylon membranes (Hybond-N, Amersham, Arlington Heights, Illinois). RNA was cross-linked to the membrane by a 5-minute exposure with a 300 nm ultraviolet transilluminator. High stringency blots were prehybridized in 0.75 M Na2HPO4 (pH 7.2), 5% sodium dodecyl sulfate, 100 μg/ml salmon sperm DNA (Sigma Chemical) for 1 hour at 65°C and then hybridized overnight in the same buffer plus the labeled probe at 65°C. Blots were then washed in 0.015 M Na2HPO4 (pH 7.2) with 1% sodium dodecyl sulfate at 65°C before autoradiographic exposure to Kodak XAR-5 film at –80°C with an intensifying screen.

Northern blots, prepared under the above high stringency conditions, show complete specificity of hybridization of the connexin probes. Connexin32 hybridizes to an mRNA of approximately 1.6 kilobases; connexion26, to approximately 2.5 kilobases; and connexion43, to approximately 3.0 kilobases.

Results

Dye Transfer

The dye transfer assays were carried out on cultures grown in parallel to those used for mRNA isolation. All of the vascular cells tested were capable of dye transfer (Table 1). Figure 1 demonstrates examples of these results for scrape-loaded human umbilical vein endothelial cells and microinjected rat aortic smooth muscle cells and bovine brain microvascular pericytes. Of the nonvascular cells tested, IMR-90 fibroblasts and AR42J were capable of dye transfer, but C6 glioma, T84, and sarcoma 180 cells (Figure 1) did not demonstrate detectable transfer when using either the scrape-load or direct microinjection assays. MDCK cells were not tested; however, MDCK cells have been shown by others not to engage in junctional transfer or to do so only to a limited extent. L929 fibroblasts were not tested in these studies; we and others have previously...
shown that these cells do not make gap junctions or engage in junctional transfer.

Northern Blots

Cultured cells. Total RNA extracts from a wide variety of cultured vascular wall cells were tested by Northern blot for connexin mRNA (Figure 2, Table 1). Message for connexin43 was detected in all vascular cell extracts including those from macrovascular and microvascular endothelium, specialized sinus endothelium, smooth muscle cells from aorta as well as mesenteric arteries, and vascular pericytes and mesangial cells.

When high stringency Northern blot analysis was used, none of the cultured vascular cells contained detectable mRNA for connexin32. Selected cultured cells were also assayed for connexin26 mRNA, with a probe that became available during the course of these studies (Table 1). No vascular cells tested contained detectable levels of this mRNA.

Comparison of vascular wall cells from different species revealed no evidence for species specificity in the expression of connexin mRNA (Table 1). Human, bovine, rat, and porcine cells contained indistinguishable message.

Of the nonvascular cell types, AR42J, MDCK, and T84 cells (all of epithelial origin) contained mRNA for connexin32 but not for connexin43 (Table 1). The mesenchymal C6 glioma, sarcoma 180, IMR-90, and L929 cells contained mRNA for connexin43 (Figure 3) but not for connexin32. Connexin26 mRNA was detected in RNA from AR42J cells but not from L929 or C6 cells.

Tissue preparations. Since connexin43 was originally isolated from heart and since connexin26 and connexin32 were originally isolated from liver, total RNA preparations from those tissues were used as positive or negative control samples for expression of the respective connexin mRNAs.

To verify that the exclusive expression of connexin43 mRNA by all of the cultured vascular cells did not present a phenotypic change due to adaptation to cell culture, we performed Northern blots of RNA extracted from fresh bovine aortic Hauthchen and rat aortic medial preparations (Figure 4). These blots revealed that both endothelium and smooth muscle also express mRNA for connexin43 in vivo. Neither preparation contained detectable connexin32 or connexin26 message (not shown).

Discussion

We have shown that the three classes of vascular wall cells (endothelial cells, smooth muscle cells, and pericytes) are all capable of intercellular communication in vitro as assessed by dye transfer assays. This confirmed our earlier results showing that certain cultured bovine and rat vascular wall cells are capable of dye transfer, as determined by the scrape-load or microinjection assays. In addition, we have now shown that human and porcine endothelial cells, rat and porcine
smooth muscle cells, and human mesangial cells are all capable of dye transfer in culture.

We have also shown that these vessel wall cells all express connexin43 gap junction mRNA. mRNAs for the other described mammalian gap junction proteins (connexin26 and connexin32) were not detectable in the high stringency blots. In addition, we found no evidence for a species specificity in results; calf, pig, rat, and human cultures contained indistinguishable message. Freshly isolated vascular tissue samples confirmed the cell culture observations since both bovine aortic endothelium (Häutchen preparation) and rat aortic smooth muscle (medial preparation) contained mRNA for connexin43 but no detectable message for connexin32 or connexin26. These findings strongly suggest that these cells communicate via gap junctional channels composed of connexin43.

What are the consequences of these different cell types expressing the same connexin? Physiological studies of different gap junctions have suggested that they have differences in unitary channel conductance and in sensitivity to possible regulators such as intracellular pH and transjunctional voltage. Sequencing of the connexins and topological studies have shown that the connexins have unique domains that are located on the cytoplasmic side of the junctional membrane where they have been proposed to serve a physiological regulatory function. Expression of the same connexin by all of the vascular cell types might allow all of their gap junctions to respond similarly under similar physiological conditions.

These studies also suggest a mechanism for gap junctional coupling between different vascular wall cells. Although the morphological evidence for heteromolecular gap junctions at the "myoendothelial" contacts between endothelial cells and pericytes or smooth muscle cells in vivo is sparse (see review in Reference 2) and other roles for these contacts have been proposed, we and others have demonstrated junctional transfer (dye, uridine nucleotides) between these cell types in vitro and in both small and large vessels in vivo. Communication between different cells might be facilitated by their expression of the same connexin. Although heteromolecular channels composed of different connexins can form in some exogenous expression systems, they are yet to be documented in vivo. Heterocellular junctional channels, composed of a single connexin, might have less structural impairments to assembly. Formation of gap junctions between all of the vascular cells would physiologically integrate them into a single functional unit.

Although we did not carry out densitometric quantitation on the Northern blot autoradiograms, it is qualitatively clear, from blots like that presented in Figure 2, that these vascular wall cells in culture contained variable but roughly similar levels of connexin43 mRNA, as a proportion of total RNA (10 μg total RNA/lane), in comparison with the apparently greater differences in expression level in vivo (Figure 4). The qualitatively large differences in the amount of mRNA for connexin43 in the Häutchen and medial samples is unexplained at present. It is
possible that large differences exist in the total amount of RNA per cell or in connexin mRNA transcription or turnover in these two cell types.

We have previously published data\textsuperscript{18} showing that junctional transfer between endothelial cells is altered in the vicinity of a denuding injury in vitro. Those data strongly suggested that turnover of junctions may be increased in cells migrating in response to the injury. Identification of the gap junction protein expressed by these cells and the new availability of connexin43-specific complementary DNA and antibody reagents will permit detailed molecular study of the role of gap junction regulation in vascular injury. Such studies may provide some understanding of the role of intercellular communication in the responses to physiological or pathological events in the vessel wall.

Is connexin43 the only gap junction protein expressed by these vessel wall cells? All of our Northern blot experiments were performed at high stringency with total RNA. It is possible that, if poly-A RNA were analyzed or if a more sensitive technique such as nuclease protection was used, we might be able to detect trace amounts of connexin26 or connexin32 mRNA. However, based on our current analyses, it is clear that they could only amount to a tiny fraction of the large amount of connexin43 mRNA expressed. A second, more likely, possibility is that these cells might express mRNAs coding for additional, as yet undiscovered, gap junction proteins. Recent molecular studies have continued to identify novel members of the connexin family.\textsuperscript{42,43} The identification of an additional connexin expressed by these cells would not invalidate the present findings but would add to the levels of complexity; in one possible scenario, cells of all vascular types could communicate via connexin43, but those of one type might have some communication specificity conferred by the expression of a second connexin.

Several cultured nonvascular cell types were tested as controls for the expression of connexin mRNA in vascular cells. In general, we found that connexin32 and connexin26 mRNAs were detectable in the tested cultures of epithelial origin whereas connexin43 mRNA was present in fibroblasts and sarcoma and glioma lines (as well as the vascular cells). None of the cell types tested contained both connexin43 and connexin32 or connexin26.

Finally, this study has raised an interesting new issue. Several nonvascular cell types (sarcoma 180, L929, \textit{C.} glioma), which communicate poorly (or not at all), expressed abundant amounts of the connexin43 mRNA (Figure 3). This finding suggests that assembly of functional gap junctions between these cells is regulated at a different step than mRNA transcription.

**Acknowledgments**

A substantial part of this work was accomplished while Dr. Beyer was working in the laboratory of Dr. D.A. Goodenough, and we are grateful for his support. The authors would also like to thank M.P. Carson, W.J. Atkinson, M.A. Gimbrone Jr., R.J. Quigg, C.F. Delp, and J.L. Madara for the gift of cell cultures. Special thanks are due to B.R. Nicholson for supplying the connexin26 clone.

**Figure 3.** Composite autoradiogram of Northern blots on samples of total RNA (10 μg/lane) from cultured nonvascular (and noncommunicating) cells with the use of a \( ^{32}P \)-labeled probe for connexin43. Lane 1, heart tissue positive control; lane 2, sarcoma 180; lane 3, \textit{C.} glioma; and lane 4, L929 fibroblasts. Arrowheads mark the positions of the 18S and 28S ribosomal RNA subunits.

**Figure 4.** Autoradiogram of Northern blot on samples of total RNA (10 μg/lane) from single-cell type tissue samples. Lane 1, heart tissue positive control; lanes 2 and 3, bovine aortic endothelial “Hütchens” preparations; and lane 4, rat aortic medial smooth muscle preparation. Arrowheads mark the position of the 18S and 28S ribosomal RNA subunits.
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Key Words: gap junctions • endothelium • vascular smooth muscle • pericytes • connexin • intercellular communication
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doi: 10.1161/01.RES.66.4.1074
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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