Differential Regulation of Gap Junctions by Proinflammatory Mediators In Vitro

Jing Hu and Ian A. Cotgreave
Division of Toxicology, Institute of Environmental Medicine, Karolinska Institute, S-171 77 Stockholm, Sweden

Abstract

The development of inflammation is an important component of host defense against infection. The cellular and molecular processes underlying inflammation are well-studied, and it is known that cells of the blood vessel wall, such as endothelial cells and smooth muscle cells, play pivotal roles. Additionally, a wide variety of proinflammatory mediators have been defined, which coordinate the multicellular processes of inflammation. Knowledge of the potential role of blood vessel gap junctional intercellular communication (GJIC) in coordinating the inflammation process, however, is limited. In this study, we report that bacterial lipopolysaccharide (LPS), as well as the proinflammatory cytokines TNF-α and IL-1β, selectively inhibit human myoendothelial GJIC in vitro without affecting GJIC between the respective homologous cell populations. This finding may represent a physiologically relevant component of the inflammatory response to infection. The work also provides some of the first clear evidence suggesting that a single eukaryotic cell can differentially regulate its GJIC between homologous and heterologous cell types in a simultaneous manner. (*J. Clin. Invest.* 1997. 99:2312–2316). Key words: connexin • cytokines • inflammation • myoendothelial channels • vascular cell

Introduction

Gap junctions are clusters of intercellular channels that provide direct cytoplasmic communication of small molecules between adjacent cells. Gap junctional intercellular communication (GJIC)[1] is thought to play an important role in the maintenance of cell differentiation and homeostasis (1). Conversely, abnormal GJIC has been proposed to be involved in the development of a number of pathophysiological conditions (2). A gap junction channel is formed by two hemichannels, or connexons, one contributed by each of the two adjacent cells. One connexon consists of six connexin (Cx) subunits. Human endothelial cells mostly express Cx43, but Cx37 and Cx40 (3, 4) are also present. Human vascular smooth muscle cells only express Cx43 and Cx40 (5, 6). Functional myoendothelial gap junctional coupling has been observed in a number of studies (7–9). Since endothelial cells are located at the interface between the blood and vascular tissue, they may act in the transfer of circulatory signals to subendothelial cells by the GJIC of second messenger molecules. Indeed, such mechanisms may be central to controlling vascular tonus (7, 8).

GJIC regulation plays a very important role in many physiological or pathophysiological processes. Little is known, however, about the role of GJIC in the inflammation process. There are a few reports suggesting that some proinflammatory mediators are involved in the regulation of GJIC (10–12). Bacterial lipopolysaccharide (LPS) has been shown to cause induction of Cx43 expression in hamster leukocytes in vitro (11), and to alter posttranscriptional regulation of Cx32 gene expression in rat liver during acute inflammation (12). IL-1α was shown to suppress GJIC in human endothelial cells (10). In this study, we demonstrate that LPS, as well as TNF-α and IL-1β, selectively inhibit human myoendothelial GJIC in vitro without affecting GJIC between the respective homologous cell populations. The work also provides some of the first clear evidence suggesting that a single eukaryotic cell can differentially regulate its GJIC between homologous and heterologous cell types in a simultaneous manner.

Methods

Cell isolation and culture. All media, serum, and materials for cell culture were obtained from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD) (Lab. Design, Stockholm, Sweden). Human umbilical vein endothelial cells (HUVECs) were prepared from umbilical cords according to Jaffe et al (13). In brief, umbilical cord veins were rinsed twice with phosphate-buffered saline (PBS) and filled with 0.1% of collagenase in PBS containing Ca^{2+} and Mg^{2+}. After incubation at 37°C for 15 min, gentle flushing of the vein released a suspension of isolated cells. The freshly isolated cells were seeded onto gelatin-coated culture dishes, and the adherent endothelial cells were grown to confluency. The cells were cultured in M199 medium supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), fungizone (1.25 mg/ml), L-glutamine (2 mM) and human serum (HS, 10%).

Human umbilical vein smooth muscle cells (HUVSMCs) were prepared by the method of Bohlin et al (14). Following the removal of endothelial cells, the umbilical cord was trimmed of the amnion, connective tissue, and the arteries, the vessel was split longitudinally and sectioned, and the pieces were placed (luminal surface down) onto a culture plate. M199 medium (as for HUVECs but supplemented with 4.5 g/liter glucose and 20% fetal calf serum [FCS] instead of HS) was added until the substrate surface was just covered. At day 5, all

Address correspondence to Ian A. Cotgreave, Division of Toxicology, Institute of Environmental Medicine, Karolinska Institute, Box 210, S-171 77 Stockholm, Sweden. Phone: 46 8 7287654; FAX: 46 8 334467; E-mail: ian.cotgreave@imm.ki.se

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1. *Abbreviations used in this paper:* Cx, connexin; GJIC, gap junction intercellular communication; HUVEC, human umbilical endothelial cell; HUVSMC, human umbilical smooth muscle cell.

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detached explants were carefully removed, and the adherent smooth muscle cells were grown to confluency. Both HUVECs and HUVSMCs were passaged by conventional trypsinization, and were used at passages two and six, respectively.

Labeling cells with PKH26 dye. The commercially available red fluorescent dye PKH26 was used to distinguish HUVSMCs and HUVECs in cocultures, largely as indicated by the suppliers. The hydrophobic PKH26 dye molecules are rapidly and stably incorporated into cellular membranes by phase-partitioning. The dye is then faithfully divided into daughter cells during karyokinesis, resulting in a 50% dilution of fluorescent intensity in these cells (15). Subconfluent cultures of HUVSMCs were labeled with PKH26 (Red Fluorescent General Cell Linker Kit; Sigma Chemical Co., St. Louis, MO). 2×10^7 HUVSMCs suspended in diluent C were mixed 1:1 with 4 μM PKH26 in diluent C, incubated at 25°C for 5 min. FCS (2 ml) was added to the sample, and was incubated for 1 min. The cell suspension was washed 3× with M199 supplemented with 10% FCS, and the cells were then resuspended in M199 supplemented with 10% HS to the desired seeding concentration. Cellular labeling (routinely 100% efficient) was examined in the suspension with a fluorescence microscope (DIAPHOT-TMD Inverted Microscope; Nikon, Tokyo, Japan) at λ_{ex} 546 nm. Once plated, control experiments demonstrated that the PKH26 dye was faithfully distributed among daughter HUVSMCs without contaminating cocultured HUVE cells.

Coculture of HUVECs and HUVSMCs. For coculture experiments, unlabeled HUVECs and PKH26-labeled HUVSMCs were mixed at a ratio of 10:1 and plated onto 35-mm, gelatin (0.1%)-coated culture dishes. Cultures were used for GJIC assay after 18–24 h of culture in M199 medium supplemented with 10% HS and 4.5 g/liter glucose. Following plating, cocultures were visualized by fluorescence microscopy (Nikon) at λ_{ex} 546 nm.

Evaluation of gap junctional intercellular communication. All the chemicals for the assay of GJIC were from Sigma Chemical Co. unless otherwise stated. Confluent HUVECs, HUVSMCs or HUVSMC/HUVEC cocultures were washed 2× with PBS, and then were exposed to LPS (30 ng/ml–3 μg/ml) (phenol extract, from Salmonella abortus equi), recombinant human TNF-α (10 ng/ml), or recombinant human IL-1β (100 pg/ml) in M199 medium, with or without 10% human serum, for between 30 min and 4 h. In some experiments, antihuman TNF-α (20 μg/ml) and/or antihuman IL-1β (working dilution 1:1,000 of commercial stock), actinomycin D (5 μg/ml) or cycloheximide (10 μM) were added to the cells 5 min before the addition of LPS. In a further test, polymyxin B (10 mg/ml in M199 medium containing 10% HS) was added to the cocultures 15 min before the addition of LPS (30 ng/ml). The efficiency of GJIC in all of these tests was then assessed after 30 min.

After the exposure of cells to the test compounds, GJIC was examined by the microinjection/dye transfer assay, according to the methods of Enomoto et al (16). In brief, glass capillaries (World Precision Instruments Inc., New Haven, CT) were prepared with a vertical pipette puller (Narishige Scientific Instrument Lab, Tokyo, Japan). Microscopically targeted cells were injected using a micromanipulator (Narishige Scientific Instrument Lab), and drawn glass capillaries were loaded with Lucifer yellow CH (10% [wt/vol] in 0.33 M LiCl). Additionally, in control studies, rhodamine B isothiocyanate-dextran (RITC-dextran, 2,000 D) was coloaded with Lucifer yellow to evaluate the potential occurrence of artifactual dye transfer during microinjection. In all cases, dye-coupled cells were examined after 5 min with a fluorescent microscope (DIAPHOT-TMD) at λ_{ex} 450–490 nm, and were photographed according to the manufacturers' recommended procedures. A total of 10 injections were made per experimental plate, and each treatment was examined in duplicate plates. The images shown are representative of 5–7 independent experiments in all cases.

Results

Using prelabeling of the HUVSMCs with the red-fluorescent probe PKH26, it was possible to select the cell type injected (15). It is evident from Fig. 1 that all of the adherent HUVSMCs were stained by the dye using the method described. Additionally, when these labeled cells were cocultured with HUVECs, cross-contamination of the endothelial cells with the dye was totally absent (Fig. 2).

Using coinjection of Lucifer yellow and RITC-Dextran in cocultures of HUVECs and HUVSMCs, both homologous GJIC between the respective cell types and heterologous myoendothelial GJIC are clearly evident (Fig. 2), without the presence of nonspecific dye coupling due to injection artifacts. When such cocultures were exposed to 30 ng/ml LPS in medium containing 10% human serum, myoendothelial GJIC was completely inhibited within 30 min, irrespective of the cell type targeted for microinjection (Fig. 3). In this manner, myoendothelial GJIC remained blocked for up to at least 4 h (data not shown). This inhibitory effect was prevented by preincubation of the cocultures with polymyxin B before the addition of LPS, indicating a role of LPS-binding protein in serum. Indeed, the omission of serum from the incubation raised the effective GJIC-inhibitory concentration of LPS to 3 μg/ml (data not shown). In accordance with the rapid nature of LPS-induced inhibition of myoendothelial GJIC, pretreatment of cells with

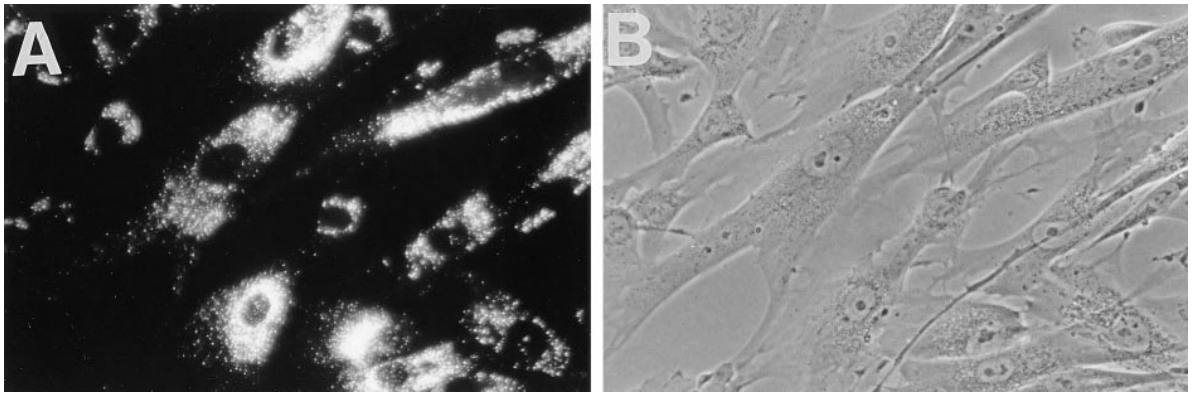

Figure 1. HUVSMCs labeling with PKH26 red fluorescent probe. (*A*) Fluorescence micrograph of the distribution of PKH26 probe in HUVSMCs at λ_{ex} 546 nm. (*B*) Phase-contrast micrograph of the HUVSMCs. The efficiency of labeling of adherent HUVSMCs is close to 100%. All the micrographs are taken of the same field (×400).

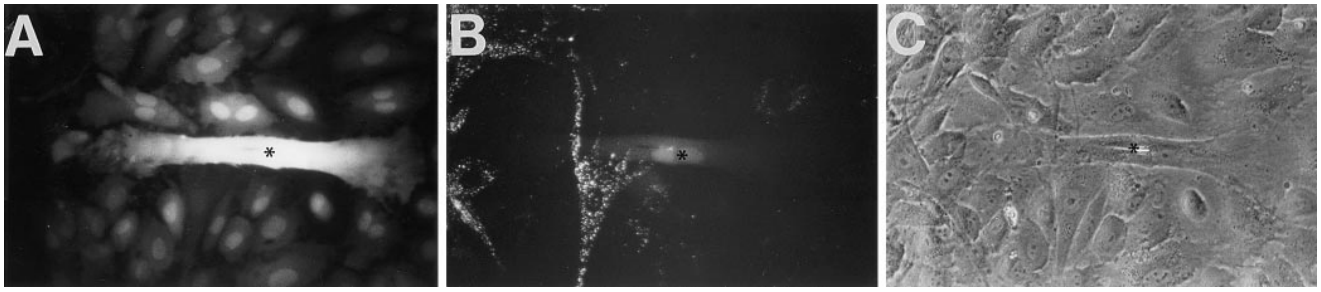

Figure 2. Homologous and heterologous GJIC between HUVECs and HUVSMCs. (*A*) Fluorescence micrograph of the distribution of Lucifer yellow from the injected HUVSMC (*) to the surrounding HUVECs and HUVSMCs at λ_{ex} 450–490 nm. Control injections routinely coupled about 30–60 HUVECs and HUVSMCs. (*B*) Fluorescence micrograph showing the staining of HUVSMCs with a PKH26 and the distribution of RITC-dextran at λ_{ex} 546 nm after coinjection with Lucifer yellow into the targeted HUVSMC (*). The lack of movement of RITC-dextran from the injected cell to surrounding cells clearly indicates a lack of nonspecific dye coupling. (*C*) Phase-contrast micrograph of the HUVEC/HUVSMC coculture. *HUVSMC injected with Lucifer yellow and RITC-Dextran. All the micrographs are taken of the same field (×400).

either actinomycin D or cycloheximide had no effect on this event (data not shown), indicating independence of de novo mRNA or protein synthesis. Interestingly, using the PKH26 probe, it is clear from Fig. 3 that homologous GJIC between HUVECs or HUVSMCs were not affected under conditions where LPS caused a total inhibition of heterologous, myoendothelial communication.

Many of the effects of LPS leading to septic shock in the organism are secondary to the overproduction of at least two cytokines, TNF-α and IL-1β (17). Thus, it is possible that these two cytokines are partially responsible for the inhibition of myoendothelial GJIC induced by LPS. In fact, LPS has been shown to trigger maximal TNF-α release within 5 to 10 min in human monocytes (18). In this study, anti–TNF-α and anti–IL-1β antibodies, added to the coculture separately or together, did not affect LPS-induced downregulation of myoendothelial GJIC (data not shown). When TNF-α or IL-1β per se were incubated with cocultures for between 30 min and 4 h, however, either TNF-α (10 ng/ml, Fig. 4) or IL-1β (100 pg/ml, Fig. 5) almost totally inhibited myoendothelial GJIC without affecting homologous GJIC between either HUVECs or HUVSMCs. Again, preincubation of the cells with either actinomycin D or cycloheximide had no effect on the inhibition caused by either cytokine (data not shown).

Discussion

Because of the technical difficulties of studying molecular events in the walls of intact blood vessels, in vitro techniques (based on the isolation of pure populations of cells and their

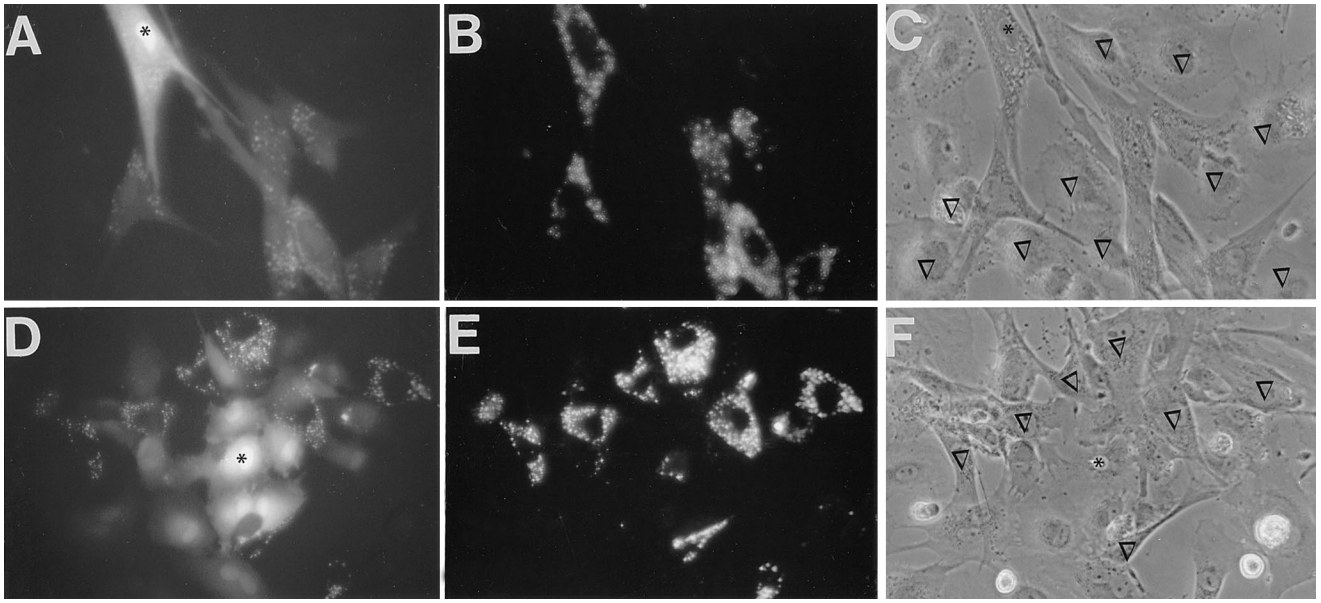

Figure 3. The selective inhibition of myoendothelial GJIC by LPS. (*A*) and (*D*) Fluorescence micrographs of Lucifer yellow distribution from an injected HUVSMC (*) to surrounding HUVSMCs only, or from an injected HUVEC (*) to surrounding HUVECs only, respectively. λ_{ex} 450–490 nm. (*B*) and (*E*) Fluorescence micrographs showing the HUVSMCs in respective cocultures in *A* and *D*, stained with PKH26 (λ_{ex} 546 nm). (*C*) and (*F*) Phase-contrast micrographs of the respective cocultures in *A* and *D*. It can be seen clearly from the paired micrographs that neither surrounding HUVECs (▽, C) nor surrounding HUVSMCs (▽, F) receive any Lucifer yellow from the injected HUVSMC (*) or HUVEC (*), respectively. All the micrographs are taken from the same fields in *A–C* and *D–F*, respectively (×400).

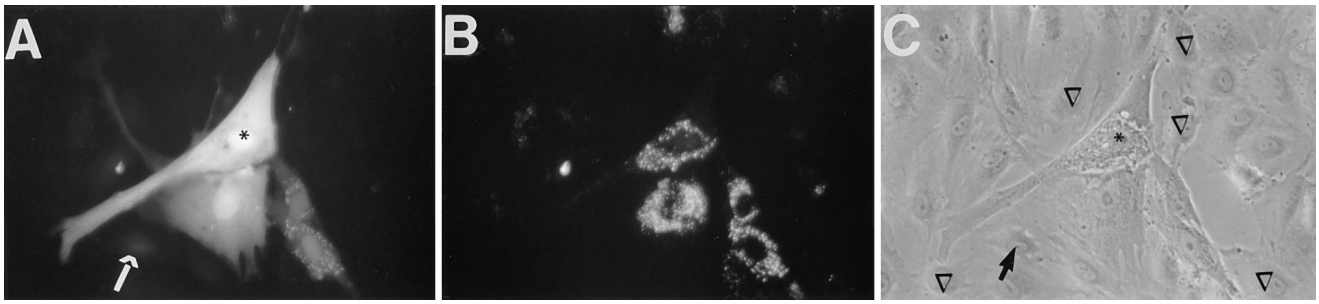

Figure 4. The inhibition of myoendothelial GJIC by human TNF-α. (*A*) Fluorescence micrograph showing that Lucifer yellow is transferred from an injected HUVSMC (*) to surrounding HUVSMCs, while only one HUVEC (→) is weakly stained. (*B*) Fluorescence micrograph showing the HUVSMCs in the coculture stained with PKH26 (λ_{ex} 546 nm). (*C*) Phase-contrast micrograph of the coculture. HUVECs surrounding the injected HUVSMC which did not receive Lucifer yellow are denoted (▽). A single, weakly stained HUVEC is marked (→). All the micrographs are taken of the same field (×400).

reconstitution of vessel components into cocultures) can be used to study myoendothelial gap junctional communication. Previous studies in vivo have yielded conflicting data concerning the efficiency of gap junctional transfer of Lucifer yellow between cells of the vascular wall. In one study, efficient transfer of the dye between smooth muscle cells was evident (19), while another indicated relatively poor smooth muscle and myoendothelial dye coupling (9). In the present in vitro study, Lucifer yellow was shown to be freely transferred via gap junctions, not only between HUVSMCs and between HUVECs, but also from HUVSMCs to HUVECs (Fig. 2). The highly efficient nature of the dye coupling in the present in vitro model may lie in the nature of the monolayer coculture system used, as the area of contact between the HUVSMCs and HUVECs is envisaged to be much greater than that in the intact vessel wall where the cells are separated by a basement membrane and the lamina propria.

Proinflammatory mediators such as LPS, TNF-α, and IL-1β exert pleiotropic effects on cells (20–22). Previous data has shown that LPS can affect posttranscriptional regulation of Cx32 in the rat liver (12). In this study, we demonstrate for the first time that these proinflammatory mediators are also capable of suppressing functional human myoendothelial GJIC in vitro. The effect of LPS on myoendothelial GJIC could be the direct consequence of the interaction between these cells and LPS, or of the autocrine effect of LPS-induced cytokines (20, 23). Addition of anti–TNF-α and anti–IL-1β, however, did not affect LPS-induced down-regulation of myoendothelial GJIC. Thus, it seems likely that LPS does not stimulate the release of these cytokines from either one or both cell types in sufficient quantities to activate their respective receptors on the cells surface.

Interestingly, the homologous GJIC between HUVECs or HUVSMCs were not affected under conditions where LPS, TNF-α, and IL-1β caused a total inhibition of heterologous, myoendothelial communication (Figs. 3–5, respectively). Irrespective of the relevance in inflammatory processes, this observation provides some of the first experimental evidence that eukaryotic cell types may simultaneously regulate their GJIC to multiple other cell types in a differential manner. This ability would be clearly advantageous in the coordination of different cellular phenotypes into complex tissues.

The origins of the differential response of homologous and heterologous GJIC between endothelial and smooth muscle cells to these proinflammatory stimuli are obscure. It is likely that the gap junctions established in a single cell to communicate with different cell populations possess different physical and/or regulatory properties, dictated primarily by their connexin compositions. The expression of multiple connexins by a single cell may provide a mechanism by which the cells regu-

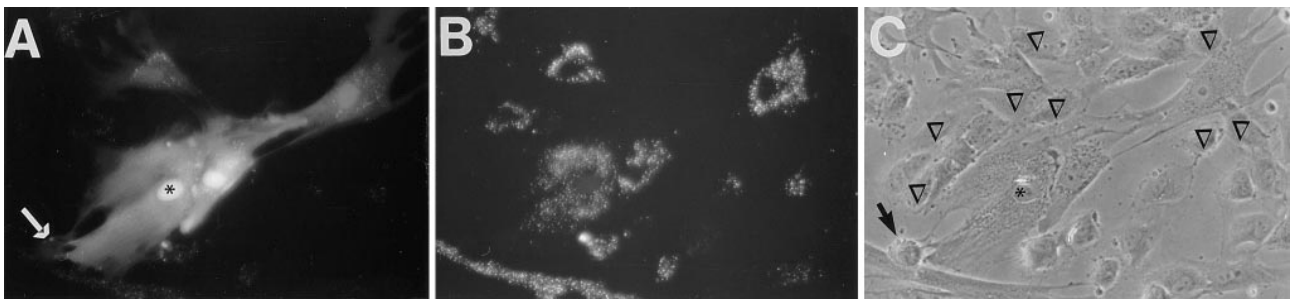

Figure 5. The inhibition of myoendothelial GJIC by human IL-1β. (*A*) Fluorescence micrograph showing that Lucifer yellow is transferred from an injected HUVSMC (*) to surrounding HUVSMCs, while only one HUVEC (→) is weakly stained. (*B*) Fluorescence micrograph showing the HUVSMCs in the coculture stained with PKH26 (λ_{ex} 546 nm). (*C*) Phase-contrast micrograph of the coculture. HUVECs surrounding the injected HUVSMC which did not receive Lucifer yellow are denoted (▽). A single, weakly stained HUVEC is marked (→). All the micrographs are taken of the same field (× 400).

late intercellular coupling through the formation of multiple channels. It has been shown that Cx37 and Cx40 can form functional gap junctional channels (3–5). Additionally, from transfection experiments, it is established that Cx43 and Cx40 are incompatible, and do not form heterotypic channels, while Cx43 and Cx40 form functional channels with Cx37 (24, 25). Thus, it is possible that myoendothelial gap junction channels studied here consist of (*a*) solely Cx43, (*b*) solely Cx40, or (*c*) mixtures of Cx37 and either Cx43 or Cx40, in which the two connexons are constructed entirely of Cx37 (from endothelial cells) and Cx43 or Cx40 (from smooth muscle cells). In the latter case, this would offer possibilities for selective responses if the gap junctions between HUVSMCs and between HUVECs were homotypic and dominated by either Cx40 or Cx43. Such differing connexin composition would allow for differential response to stimuli to be achieved by varying the pattern of post-translational modification of the connexin proteins themselves. Thus, it has been shown that distinctly different types of gap junction channels expressed in connexin-transfected SKHep1 cells exhibit different behavior under similar phosphorylation conditions (26).

The reconstitution of critical cellular components in an in vitro coculture system is not wholly representative of the vessel in vivo. The modulation of myoendothelial GJIC by LPS and proinflammatory cytokines in vitro however, throws new light onto a possible role for altered GJIC in the development and maintenance of the human inflammatory response. It may be speculated that the inhibition of myoendothelial GJIC may alter signal transduction pathways coupling endothelial cells and smooth muscle cells into a functional unit (7, 8). Additionally, such a blockade may spare the need for GJIC within the vessel wall at a time of increased endothelial permeability during the development of tissue edema.

Acknowledgments

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