Alteration in the gene expression pattern of primary monocytes after adhesion to endothelial cells

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Monocytes originate from precursors made in the bone and remain in the circulation for nearly 24 h. Much effort has been done to identify the molecules regulating transendothelial migration of monocytes during inflammatory conditions. In contrast, considerably less is known about the process of constitutive monocyte emigration although nearly 340 million monocytes leave the circulation each day in healthy individuals. Previous studies indicated that chemokines were up-regulated in monocytes cocultured with endothelial cells that induce the retraction of the latter cell type, thereby increasing vascular permeability. Thus, we hypothesized that the utilities required for efficient constitutive monocyte extravasation are generated by monocytes themselves because of adhesion to naïve endothelial cells. To test this hypothesis, cDNA microarray analysis was performed to determine the changes in the gene expression pattern of primary monocytes that have been attached to endothelial cells compared with monocytes that were held in suspension, and we were able to identify three major groups of genes. The first group includes genes such as matrix metalloproteinase 1, monocyte chemoattractant protein 1, and tissue transglutaminase 2, which are likely required for monocyte extravasation. The second group consists of genes that are expressed in phagocytes such as caveolin-1 and CD74. Finally, the third group comprises genes that are expressed in cells of endothelial tissue and cartilage including E-selectin, fibronectin-1, matrix Gla protein, and aggrecanase-2. In summary, we conclude that adhesion of peripheral blood monocytes to naïve endothelial cells has two effects: mandatory extravasation-specific genes are regulated, and the differentiation program of monocytes is initiated.

differentiation | cDNA microarray

Monocytes belong to the mononuclear phagocyte system. They derive from hematopoietic stem cells in the bone marrow and circulate in the blood for ≈ 24 h or more before extravasation into the connective tissue (1). Once monocytes have entered the subendothelial connective tissue they differentiate into distinct phagocytes including dendritic cells and tissue macrophages. The regulation of transendothelial migration of monocytes during inflammatory conditions is well recognized. Activation of tissue macrophages by invaded pathogens results in the production of proinflammatory cytokines such as TNF- α and IL-1 β and various chemokines including monocyte chemoattractant protein 1 (MCP-1) and IL-8 (2, 3). Proinflammatory cytokines activate the endothelium, thereby inducing the up-regulation of the adhesion molecule E-selectin (4), which is involved in the conversion of leukocyte rolling to firm adhesion to the endothelium (5) and increases vascular permeability (6). The permeability of the endothelial lining is enhanced as well by the chemokines MCP-1 and IL-8 (7, 8); however, the predominant function of these factors is the direction of transendothelial migration of leukocytes including monocytes (9–11) and guidance through the tissue to the area of inflammation.

In contrast to the well known process of monocyte emigration during inflammatory conditions, considerably less is known about the molecules regulating the trafficking of monocytes, particularly the constitutive trafficking of monocytes across the endothelial lining and through tissues in the absence of infection (12). For humans the number of constitutively emigrating monocytes was estimated to be ≈ 340 million cells per day (13). The role of constitutive monocyte emigration is unclear, but it is possible that this mechanism is essential for providing a pool of tissue macrophages and dendritic cells.

A few studies have indicated that chemokines such as macrophage inflammatory protein 1 α (14), IL-8 (15), and MCP-1 (15, 16) are up-regulated in monocytes that have been cocultivated with naïve endothelial cells. Thus, it is possible that the utilities required for efficient constitutive monocyte extravasation are generated by monocytes themselves once they adhere to endothelial cells.

To test this assumption we analyzed the gene expression pattern of peripheral blood monocytes that had been cocultivated with naïve human umbilical vein endothelial cells (HUVECs) by cDNA microarray analysis. Primary monocytes that were held in suspension culture served as a control. We were clearly able to identify differentially expressed genes in HUVEC-attached monocytes known to be involved for transendothelial migration and differentiation into macrophages such as MCP-1, matrix metalloproteinase 1 (MMP-1), or tissue transglutaminase 2 (tTG-2), and caveolin-1, CD74 and CD64, respectively. Moreover, we identified up-regulated genes in HUVEC-attached monocytes, which are known to be expressed by endothelial cells such as E-selectin, fibronectin-1, or connective tissue growth factor (CTGF) and cartilage such as matrix Gla protein and aggrecanase-2.

In summary, we conclude from our data that adhesion of peripheral blood monocytes to naïve endothelial cells has two effects. First, genes are regulated that are required for the subsequent transendothelial migration step. Second, the differentiation program of monocytes into phagocytes and other lineages is initiated.

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Abbreviations: CTGF, connective tissue growth factor; HUVEC, human umbilical vein endothelial cell; MCP-1, monocyte chemoattractant protein 1; MMP-1, matrix metalloproteinase 1; RISH, RNA *in situ* hybridization; ICC, immunocytochemistry; tTG-2, tissue transglutaminase 2.

Data deposition: The microarray data have been deposited in the ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-854).

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Table 1. Up-regulated phagocyte and mesenchymal genes in HUVEC-attached monocytes

Accession no./ clone ID	Gene	Fold change	Predominant expression
M15330	IL-1 β	14.139	Phagocyte lineage
M13509	MMP-1	8.676	Phagocyte lineage
M98479	tTG-2	5.830	Phagocyte lineage
M27024	LPS-associated protein 2	5.166	Phagocyte lineage
M24545	MCP-1	5.013	Phagocyte lineage
M13560	CD74 invariant chain	5.013	Phagocyte lineage
X06820	rhoB	4.810	Phagocyte lineage
Z18951	Caveolin-1	4.728	Phagocyte lineage
X15606	ICAM-2, cell adhesion ligand for LFA-1	3.321	Phagocyte lineage
M63835	IgG Fc receptor I (CD64)	3.021	Phagocyte lineage
IMAGE:127269	Chemokine (C-C motif) ligand 15	2.745	Phagocyte lineage
J03225	Tissue factor pathway inhibitor	2.550	Phagocyte lineage
X68277	Dual specificity phosphatase-1	2.527	Phagocyte lineage
V01512	Cellular oncogene c-fos	2.323	Phagocyte lineage
X53331	Matrix Gla protein	27.523	Mesenchymal lineage
IMAGE:154494	Insulin-like growth factor binding protein 4	16.057	Mesenchymal lineage
X83703	Ankyrin repeat domain 1 (cardiac muscle)	8.415	Mesenchymal lineage
M30640	E-selectin	7.439	Mesenchymal lineage
X78947	Connective tissue growth factor	6.361	Mesenchymal lineage
IMAGE:44477	VCAM-1	5.895	Mesenchymal lineage
X02761	Fibronectin-1	4.035	Mesenchymal lineage
W73702	Aggrecanase-2	3.157	Mesenchymal lineage
AF043045	Actin-binding protein ABP-278 (filamin B, β)	2.406	Mesenchymal lineage
M57730	Ephrin-A1	2.104	Mesenchymal lineage
J02973	Thrombomodulin	2.035	Mesenchymal lineage

Fold change represents the mean of five independent experiments. All regulated genes are shown in [SI Table 2](#).

Results

The Gene Expression Pattern of Peripheral Blood Monocytes Is Altered After Adhesion to a HUVEC Monolayer. Of 7,200 spotted cDNAs, 56 genes were found to be regulated significantly ($P < 0.05$) in monocytes cocultivated for 2 h with endothelial cells, 36 genes were found to be up-regulated, and 20 genes were found to be down-regulated [supporting information (SI) Table 2]. Genes were categorized according to cell types of predominant expression. Sixteen genes (14 up-regulated and two down-regulated) belong to the phagocytic lineage (Table 1), whereas 13 genes (11 up-regulated and two down-regulated) belong to cell types of the mesenchymal lineage (Table 1). Genes belonging to the hematopoietic lineage (seven genes, all down-regulated) and noncategorized genes (designated as misc; 20 genes: 11 up-regulated and nine down-regulated) are summarized in SI Table 2.

Control experiments with monocytes that adhered to a plastic surface showed a completely different gene expression pattern. Of 7,200 spotted cDNAs, 70 genes were found to be regulated significantly ($P < 0.05$); 16 genes were up-regulated, and 54 genes were down-regulated (SI Table 3). None of these genes belong to the mesenchymal lineage. Only two genes were found to be regulated in both HUVEC-attached and plastic-attached monocytes. However, the dual specificity phosphatase 1 was up-regulated in HUVEC-attached monocytes (Table 1) but down-regulated in plastic-attached monocytes (SI Table 3). In contrast, the pro-platelet basic protein CTAP-3 was down-regulated in both HUVEC-attached and plastic-attached monocytes.

Validation of Endothelial Cell-Related Genes: E-selectin. E-selectin expression in HUVEC-attached monocytes was determined on both a protein level and an mRNA level by immunocytochemistry (ICC) and combined ICC and RNA *in situ* hybridization (RISH) because these methods allow for discrimination between monocytes and HUVECs on a cellular level. E-selectin protein expression was clearly detectable in a small subset of HUVEC-

attached monocytes, but not in monocytes that were held in suspension (Fig. 1A). Thus, monocyte-derived E-selectin is localized in the cytoplasm of the cells (Fig. 1A*b*, arrows), which was additionally verified by an *xz* scan (data not shown). A colocalization with CD14 on the surface of monocytes was not observed.

Similar results were obtained for the combined ICC plus RISH strategy. E-selectin mRNA was clearly detectable in HUVEC-attached monocytes (Fig. 1*Ad*), but not in suspension monocytes (Fig. 1*Ac*), thus indicating that E-selectin is definitely expressed in HUVEC-attached monocytes. Control experiments with the specific E-selectin sense probe were negative (data not shown). Flow-cytometry analysis revealed that, on average, 2.19% of HUVEC-attached monocytes were E-selectin-positive (Fig. 1*B*). Thus, E-selectin is definitely expressed in single monocytes after adhesion to endothelial cells.

Validation of cDNA Microarray Data. *CTGF* and *fibronectin-1*. Expression of CTGF and fibronectin-1 was detectable on both an mRNA level and a protein level in HUVEC-attached monocytes and, to a much lesser extent, also in suspension monocytes. The average number of CTGF and fibronectin-1-positive HUVEC-attached monocytes was ≈ 5 –10%, and 1–5% for control monocytes, respectively. CTGF is localized beneath the plasma membrane of both HUVEC-attached monocytes and control cells (Fig. 2 *Aa*, arrowheads, and *Ab*, arrows). Compared with suspension monocytes the CTGF expression level is much higher in HUVEC-attached monocytes (Fig. 2*A*), indicating a CTGF up-regulation, which is supported by combined ICC plus RISH (Fig. 2 *Ac* and *Ad*). Control experiments using a specific CTGF sense probe were negative (data not shown).

Fibronectin-1 expression is found on the surface of monocytes cocultured with endothelial cells (Fig. 2*Bb*). Moreover, compared with E-selectin and CTGF the expression level of fibronectin-1 is much higher, and it appears that fibronectin-1-expressing HUVEC-

Fig. 1. E-selectin is up-regulated in a small subset of HUVEC-attached monocytes. (A) ICC (a) and combined ICC plus RISH (c) show that E-selectin expression is not detectable in suspension monocytes. In contrast, E-selectin protein (b) and E-selectin mRNA (d) are detectable in HUVEC-attached monocytes. CD14 expression of monocytes appears in red, and E-selectin expression (on both a protein level and an mRNA level) is shown in green. Arrows indicate the intracellular presence of E-selectin expression in HUVEC-attached monocytes. (Scale bars: 20 μm .) (B) Verification of E-selectin up-regulation in HUVEC-attached monocytes by flow cytometry. The expression of the molecule of interest is shown as a shaded histogram. Open histograms represent staining with isotype-matched control monoclonal antibodies. On average $2.19 \pm 1.25\%$ of HUVEC-attached monocytes were positive for E-selectin expression. The results shown are representative of at least three independent experiments.

least three independent experiments.

attached monocytes are coated properly with this protein (Fig. 2*Bb*, arrows). The observation that fibronectin-1 is expressed in both cell fractions was further validated by combined ICC and RISH (Fig. 2*Bd* and *Be*). Experiments with the specific fibronectin-1 sense probe were negative (data not shown). CD14 protein expression in fibronectin-1-positive cells by ICC is rather low or even not detectable in contrast to combined ICC and RISH. This effect is likely attributed to the additional monocyte separation step, which is included in the combined ICC plus RISH protocol and which includes a trypsin/EDTA digestion step. It is well recognized that fibronectin-1 is degraded by trypsin (17) and thus is removed from the surface of fibronectin-1-expressing monocytes.

MMP-1 and tTG-2. tTG-2 is localized in the plasma membrane of both suspension monocytes and HUVEC-attached monocytes (Fig. 3*Aa*, arrowheads, and *Ab*, arrows). Both tTG-2 expression level and the number of tTG-2-positive HUVEC-attached monocytes are increased as compared with suspension cells. Similar results were obtained by combined ICC and RISH (Fig.

Fig. 2. CTGF and fibronectin-1 expression is up-regulated in a subset of HUVEC-attached monocytes. (A) CTGF expression is detectable on both a protein level (a and b) and an mRNA level (c and d) in suspension and HUVEC-attached monocytes. However, the CTGF protein expression level is higher in HUVEC-attached monocytes (b, arrows) as compared with control cells (a, arrowheads) indicating CTGF up-regulation. (B) Fibronectin-1 expression is detectable on both a protein (a and b) and mRNA (c and d) in suspension and HUVEC-attached monocytes. However, the fibronectin-1 protein expression level is higher in HUVEC-attached monocytes (b, arrows) as compared with control cells (a, arrowheads) indicating fibronectin-1 up-regulation. CD14 expression of monocytes appears in red, and CTGF and fibronectin-1 expression (on both a protein level and an mRNA level) is shown in green. (Scale bars: 20 μm .) The results shown are representative of at least three independent experiments.

Discussion

In the present study we investigated the changes in the gene expression profile of primary monocytes after constitutive adhesion to endothelial cells. The rationale of this study was given by the fact that constitutive emigration of leukocytes/lymphocytes is a common phenomenon in the living body. For instance, B and T cells recirculate continuously through secondary lymphoid organs (18). However, until now considerably less has been known about the molecules regulating the constitutive trafficking of lymphocytes and leukocytes across the endothelial lining (12). For instance, the endothelium is activated by proinflammatory cytokines such as TNF- α and IL-1 β (19). Activated endothelial cells up-regulate the expression of adhesion molecules such as E-selectin and chemokines including MCP-1 and

Fig. 3. tTG-2, but likely not MMP-1, is up-regulated in HUVEC-attached monocytes. (A) tTG-2 expression is detectable on both a protein level (a and b) and an mRNA level (c and d) in suspension and HUVEC-attached monocytes, and tTG-2 is more highly expressed in HUVEC-attached monocytes. tTG-2 is localized in the plasma membrane of both control (a, arrowhead) and HUVEC-attached (b, arrows) monocytes. (B) The expression levels of MMP-1 appear to be similar in suspension (a and b) and HUVEC-attached (c and d) monocytes. Like tTG-2, MMP-1 is found in the plasma membrane of monocytes (a, arrowheads; b, arrows). CD14 expression of monocytes appears in red, and tTG-2 and MMP-1 expression (on both a protein level and an mRNA level) is shown in green. (Scale bars: 20 μm.) The results shown are representative of at least three independent experiments.

IL-8 (4). E-selectin is involved in the recruitment and firm adhesion of leukocytes to endothelial cells (2, 3), whereas IL-8, MCP-1, and other chemokines are presented on the surface of endothelial cells and are critical for transmigration of neutrophil granulocytes and monocytes (9–11).

Our data show that various genes (56 of 7,200) were regulated significantly ($P < 0.05$) in HUVEC-attached monocytes within a time frame of 2 h. Depending on the cell type of predominant expression, the identified genes were categorized into genes belonging to the phagocytic, mesenchymal, and hematopoietic lineages. However, all genes belonging to the hematopoietic lineage were found to be down-regulated in monocytes after adhesion to endothelial cells, but the reasoning for that remains unclear.

Our data clearly show that monocytes up-regulate and express mesenchymal cell-related genes such as E-selectin (20), fibronectin-1 (21), and CTGF (22) after adhesion to endothelial cells. This was validated on a cellular level by ICC and combined ICC and RISH. The finding that monocytes express mesenchymal genes including endothelial genes such as E-selectin (20), fibronectin-1 (21), and CTGF (22) as well as cartilage genes including matrix Gla protein (23) and aggrecanase-2 (24) is in agreement with recent reports demonstrating a phenotypic overlap between endothelial cells and monocytes (25, 26). For instance, Rohde *et al.* (27) showed that primary monocytes already express endothelial cell-specific markers such as vascular endothelial cell-cadherin (VE-cadherin; CD144) and endoglin (CD105), which substantiate the close relationship between

Fig. 4. Schematic overview of the changes in the gene expression profile of HUVEC-attached monocytes. During constitutive adhesion to endothelial cells, the gene expression pattern of primary monocytes is altered and genes are regulated that are required for subsequent transendothelial migration. Additionally, the differentiation program of monocytes into phagocytes and other lineages is initiated.

these two cell types. Additionally, Cipolletta *et al.* (28) recently showed that circulating CD14⁺ monocytes express VCAM-1, which is congruent with our data (Table 1). In addition to the phenotypic overlap between monocytes and endothelial cells, two recent studies indicated that a subset of human peripheral blood monocytes can differentiate into several distinct mesenchymal lineages (29) as well as into T lymphocytes, epithelial cells, neuronal cells, and liver cells (30). *In vitro* studies of Harraz *et al.* (31) showed that a subset of peripheral blood-enriched CD14⁺ CD34⁺ monocytes can take on an endothelial cell-like phenotype in culture. The authors concluded from their findings that monocytes differentiate into macrophages, dendritic cells, or endothelial cells depending on environmental cues, which could include soluble factors, extracellular matrix interactions, an appropriate three-dimensional lattice, or specific cell–cell interactions (31). This in turn is in agreement with the current concept of trans-differentiation, in which the instructions for trans-differentiation are given by the local tissue milieu itself (32). Furthermore, this suggestion would be in agreement with our data indicating that one environmental cue might be the endothelium itself. Only cocultivation with endothelial cells resulted in an up-regulation of endothelial cell-specific genes in monocytes. Monocytes that were adhered to a plastic surface did not up-regulate genes that belong to the mesenchymal lineage. Only phagocyte-related genes were regulated (SI Table 3). These findings indicate that the specific cell–cell interactions between endothelial cells and monocytes might be crucial for up-regulation of endothelial cell-specific genes in a subset of HUVEC-attached monocytes (Fig. 4).

Regulated genes in HUVEC-attached monocytes belonging to the phagocytic lineage include MCP-1 (16), MMP-1 (33), tTG-2 (34), CD74 (35), and caveolin-1 (36, 37). The up-regulation of MCP-1 by monocytes cocultured with endothelial cells was already reported by other groups (15, 38) and was therefore used as a positive control. MMP-1 is required for degradation of the basal lamina (33), which is a prerequisite for monocyte emigration. Additionally, Akimov and Belkin (34) showed recently that cell surface-bound tTG-2 served as an integrin-associated adhesion receptor that is involved in the adhesion of monocytes to fibronectin. Because endothelial cells express fibronectin (39), the up-regulation of tTG-2 by monocytes might be required for adhesion to endothelial cells. An antisense-based down-regulation of cell surface tTG-2 or interference with the adhesive function significantly decreased the

adhesion of monocytes to fibronectin and markedly reduced the migration of monocytes on fibronectin-1 (34). It was also shown that expression of tTG-2 increases drastically during monocyte differentiation into macrophages (34). Thus, adhesion to the endothelium does not only regulate genes that are required for subsequent transendothelial migration but might also initiate the differentiation program of monocytes. This would be in agreement with the finding that HUVEC-attached monocytes showed an up-regulation of CD74 (35), caveolin-1 (36, 37), and CD64 (40). CD74 (invariant chain) is required for antigen presentation on major histocompatibility complex class II molecules (35). Caveolin-1 and caveolin-2 have been identified as providing a novel route by which several pathogens are internalized by antigen-presenting cells (36, 37). CD64 (Fc- γ -RI) is constitutively expressed on monocytes and represents a high-affinity Fc- γ receptor that plays a pivotal role in the immune response (40). A schematic overview is given in Fig. 4.

In summary, our data indicate that during constitutive adhesion to endothelial cells the gene expression pattern of primary monocytes is altered and genes are regulated that are required for subsequent transendothelial migration and that the differentiation program of monocytes into phagocytes and other lineages is initiated.

Methods

Isolation of HUVECs. HUVECs were isolated from freshly dissected umbilical cords as described previously (41). HUVECs were maintained in endothelial cell growth medium ECGM (Promocell, Heidelberg, Germany) containing 2% FCS, 0.4% endothelial cell growth supplement/heparin, 0.1 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor, and 1 μ g/ml hydrocortisone (SupplementMix; Promocell). HUVECs were used for experiments after they were confluent for 2 days. All cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Isolation of Peripheral Blood Mononuclear Cells by Negative Selection. Peripheral blood mononuclear cells were isolated from 10 different healthy volunteer donors by Ficoll-Hypaque (PAA, Linz, Austria) density-gradient centrifugation. Monocyte negative selection was performed via magnetic activated cell sorting using the Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purity of negatively isolated monocytes was determined by flow cytometry (FACSCalibur; Becton Dickinson, Heidelberg, Germany) by using an anti-CD14-FITC antibody (Leinco Technologies, St. Louis, MO) and the appropriate isotype control (IgG2a-FITC; Immunotech, Marseille, France) and was >96% in each experiment.

Adhesion Assay. Negatively isolated monocytes were divided in two fractions, and one half was coincubated for 2 h with confluent HUVECs. In control experiments negatively isolated monocytes were adhered to a plastic surface for 2 h. The other fraction served as a control population kept for 2 h in suspension. Both fractions were held in a humidified atmosphere with 5% CO₂ at 37°C. To prevent induction of monocyte differentiation due to adhesion on plastic, tubes were precoated with Repel-Silane (Sigma, Taufkirchen, Germany). Tubes were washed twice with 70% ethanol followed by thorough washing with PBS to remove alcohol.

Positive Selection of Monocytes. After incubation (2 h), the supernatant of the monocyte HUVEC cocultivation assay was removed, and control cells (suspension culture) were transferred into a new tube and centrifuged (310 $\times$ g at room temperature for 10 min). Subsequently, all samples were treated with 0.25% Trypsin/EDTA (Sigma) at 37°C for a maximum of 10 min until

cocultivated cells detached from the culture flask. Cocultured monocytes were isolated by positive selection by using the CD14 MicroBeads kit (Miltenyi Biotec) according to the manufacturer's protocol. The control population (suspension cell culture) received the same treatment. The purity of the separated CD14⁺ cells was determined by morphological criteria such as the cell size (monocytes, 12–20 μ m; HUVECs, 25–50 μ m) and the granularity and was found to be >98%.

Microarray Procedures. DNA microarray production and analysis of gene expression were performed as described recently (42). In brief, the used cDNA microarray chip contained 7,200 sequence-verified human cDNA clones (German Resource Center for Genome Research, Berlin, Germany), which were annotated by using the SOURCE database (43). Total RNA was isolated by using the Qiagen RNeasy (Qiagen, Hilden, Germany) system according to the manufacturer's protocol. RNA conversion into cDNA, dye coupling, and hybridization were performed as described (42).

Data Analysis. Data from scanned arrays were obtained by using the GenePix software package (Axon Instruments, Union City, CA). Cy5-to-Cy3 ratios of medians generated by the GenePix software for each gene (spot) were intensity-dependent normalized. Genes (spots) that did not pass the filter criteria [spot size ≥ 60 μ m, Flag (as in GenePix) = 0, signal-to-noise ratio in at least one channel ≥ 3.0] were eliminated. Microarray studies were confronted with issues of multiple statistical testing of a large number of genes (in the thousands) in a much smaller number of samples (e.g., three per time point). The obtained cDNA microarray data were analyzed by two methods: (i) an empirical setting of statistical thresholds for fold changes between samples, which is most widely used in published studies (a >2-fold up-regulation or down-regulation was assumed to be significant); and (ii) the Null hypothesis, whereby it is assumed that all genes are not differentially expressed. Thus, a normal distribution of log ratios is assumed. After calculation of the mean and standard deviation of ratios, a *P* value was computed for each ratio. Calculations were performed by using Microsoft Excel. *P* < 0.05 was considered to be significant.

ICC. Measurements were performed by using an inverse confocal laser scanning microscope (Leica TCS 4D; Leica, Bensheim, Germany). Monocytes held in suspension and HUVEC-attached monocytes were cultured for 2 h as described above. Cells were fixed with 0.5% paraformaldehyde for 2 min at 4°C, washed once with HBSS/0.5% BSA, and permeabilized with saponin [0.01% (wt/vol) in HBSS/0.5% BSA]. Before antibody staining, suspension monocytes were cytospun on polylysine-coated slides. Suspension cells and cocultured cells were double-stained for CD14 expression and the appropriate target protein expression for 30 min using the following antibodies: anti-CD14-PE-Cy5 (clone RMO52; Beckman Coulter, Krefeld, Germany), anti-CD62E-FITC [clone CI26CI0B7; Bender MedSystems, Vienna, Austria (44)], anti-human fibronectin-1, anti-CTGF, anti-tTG-2, and anti-MMP-1 (all antibodies are purified IgG polyclonal rabbit IgG and were purchased from BIOZOL Diagnostica, Eching, Germany). Primary polyclonal rabbit antibodies were stained with goat anti-rabbit IgM+IgG-FITC (BIOZOL Diagnostica). Isotype-matched antibodies served as a control: IgG2a-PE-Cy5 (BD Pharmingen), IgG2a-FITC (Immunotech), and normal rabbit IgG (Santa Cruz Biotechnology, Heidelberg, Germany). Samples were fixed again with 0.5% paraformaldehyde at 4°C.

Combined RNA *In Situ* Hybridization and ICC. The combined RISH plus ICC was performed in accordance with Speel *et al.* (45) with modifications. Negatively isolated monocytes were cocultured

with HUVECs or in suspension and separated as described above. Monocytes were fixed with 3.7% formaldehyde and washed twice with PBS before they were cytospun on polylysine-coated slides. Control cells received the same treatment. Slides were blocked with PBS-Tween-NGS [PBS, 0.05% (vol/vol) Tween 20, and 2–5% normal goat serum (Sigma)], incubated for 45 min with horseradish-peroxidase conjugated goat anti-mouse-antibody (Dianova, Hamburg, Germany), and washed once with PBS-Tween 20 [PBS and 0.05% (vol/vol) Tween 20]. CD14 expression of monocytes was stained with SIGMA FAST fast red TR/naphthol AS-MX phosphate (Sigma) for 5–15 min at 37°C. Reaction was stopped by washing three times with PBS. Samples were digested with pepsin (100 μ g/ml in 10 mM HCl) for 10–20 min at 37°C. After washing twice with PBS and twice with 2 $\times$ SSC, samples were refixed with 1% paraformaldehyde. Cells were dehydrated with 70%, 90%, and 100% ethanol (3 min each, room temperature) and covered with 100 μ l of probe solution [3.5 $\times$ SSC, 0.1 μ g/ μ l human Cot1-DNA (Roche, Mannheim, Germany), 0.01 μ g/ μ l polyA-RNA (Invitrogen, Karlsruhe, Germany), 0.2 μ g/ μ l yeast t-RNA (Sigma), and 2 μ l of probe (200 ng/ml)]. Specific antisense and sense digoxigenin-RNA-labeled probes were generated by *in vitro* transcription by using digoxigenin-RNA labeling mix and T3 RNA-polymerase and T7

RNA-polymerase, respectively, according to the manufacturer's instructions (Roche). PCR products from bacterial clones (Deutsches Ressourcenzentrum für Genomforschung) served as templates. Samples were hybridized overnight at 64°C and subsequently washed four times for 5 min with 2 $\times$ SSC. Thereafter, samples were washed once with PBS and blocked with blocking solution (Roche) for 30 min. Samples were washed once with buffer A (100 mM Tris-HCl, pH 7.4/125 mM NaCl), and digoxigenin-RNA was detected by a 1-h incubation with the appropriate FITC-conjugated anti-DIG-antibody (Dianova). Subsequently, samples were washed three times for 10 min with buffer A and analyzed by using an inverse confocal laser scanning microscope (Leica TCS 4D; Leica).

Flow-Cytometry Analysis. Measurements were performed by using a FACSCalibur flow cytometer (Becton Dickinson). Monocytes were cultured and stained for CD14 and E-selectin expression as described above. However, cocultured cells were separated by 0.25% Trypsin/EDTA (Sigma) treatment (10 min, 37°C) before the staining procedure. Suspension cells received the same treatment.

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