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ATTENUATION OF HUMAN LEUKOCYTE ADHERENCE TO ENDOTHELIAL CELL MONOLAYERS BY TYROSINE KINASE INHIBITORS

Patrick E. McGregor, Devendra K. Agrawal, and John D. Edwards*

Creighton Vascular Center, Department of Surgery
Creighton University School of Medicine, Omaha, NE 68178

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Expression of cell adhesion molecules is controlled by various cytokines including interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα), but the underlying mechanisms are not clear. In this study, we investigated the role of tyrosine phosphorylated proteins in the regulation of leukocyte adhesion to endothelial cells. We examined the effect of genistein and herbimycin-A, selective inhibitors of tyrosine kinase, on the adherence of human blood neutrophils, lymphocytes, and monocytes to monolayers of human umbilical vein endothelial cells. Both genistein and herbimycin A significantly inhibited IL-1 and TNFα-induced upregulation of neutrophils ($p < 0.05$) and monocyte ($p < 0.01$) adherence. IL-1 and TNFα-stimulated lymphocyte adherence was diminished in the presence of herbimycin A ($p < 0.05$), but genistein only inhibited TNFα-stimulated adherence. There was no significant effect of genistein on IL-1-induced lymphocyte adherence. These novel findings reveal for the first time that tyrosine phosphorylated proteins may regulate leukocyte adherence and cell adhesion molecule expression on the endothelium.

The inflammatory response is a key element in the pathogenesis of certain vascular diseases following endothelial disruption and injury. Integral to the inflammatory response is the margination, adherence, and migration of leukocytes towards the endothelium (1). Acute inflammatory conditions enhance the chemotaxis and margination of polymorphonuclear leukocytes (PMNs), whereas in chronic inflammatory conditions lymphocytes and monocytes migrate towards and accumulate at the inflammatory site. Inflammatory cells also activate endothelial cells and release cytotoxic mediators (2). Adhesion molecules (intergrins and selectins) are an essential component of cell to cell recognition during this process (3). Very recently cytokines such as IL-1 and TNFα have been implicated in the regulation of cell adhesion molecule expression. The resultant

*Corresponding Author. FAX: (402)280-4593.

Abbreviations: EDTA, ethylene diamine tetraacetic acid; FCS, fetal calf serum; HUVEC, Human umbilical vein endothelial cell; IL-1, Interleukin-1; PMN, Polymorphonuclear leukocytes; RBC, red blood cell; TNFα, Tumor necrosis factor-alpha; WBC, white blood cell.

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inflammatory millieu at the sites of vascular injury sets the stage for both cellular damage and cellular proliferation.

Both endothelial and white blood cells have the biochemical machinery and capacity to generate molecular signals such as the cytokines, IL-1 and TNFα. These mediators, released by the activated WBCs, accumulate at the site of injury. These agents modulate the activity of cell adhesion molecules either via upregulating their receptors on fibroblasts, macrophages, and epithelial and endothelial cells, and/or via enhancing the receptor activity by affecting a site beyond the receptor site, such as protein kinases, tyrosine kinases, etc.

In this study, we examined the role of tyrosine phosphorylated proteins in the upregulation of cell adhesion molecules on endothelial cells. We have examined the effect of genistein and herbimycin A, selective inhibitors of tyrosine kinase, on the adherence of human blood neutrophils, lymphocytes, and monocytes to HUVEC monolayers.

MATERIALS AND METHODS

Isolation and Culturing of HUVEC: Human umbilical vein endothelial cells (HUVEC) were harvested from human umbilical cords, stored in cord buffer (0.137 NaCl, 0.027 KCl, 0.01 phosphate buffer, 0.011 glucose; pH 7.35) for up to 48 hr, followed by treatment with 0.1% collagenase (Type IA, SIGMA) by the method established in our laboratory as described elsewhere (4). The isolated endothelial cells were grown in T-25 flasks in M-199 medium containing 20% fetal calf serum, endothelial cell growth supplement 67 mg/L, heparin 15,000 U/L, penicillin/streptomycin 10,000 U/L, and Fungizone 2.5 mg/L.

Leukocyte Separation: White blood cells from human blood were isolated and purified by the method reported previously by Numao and Agrawal (5). Briefly, 40 ml of venous blood was collected from healthy human volunteers in a tube containing 2% EDTA. RBCs were sedimented by using 6% dextran for 90 min at 37°C. The leukocyte-rich plasma was collected and diluted with PIPES buffer and centrifuged. The cell pellet of WBCs was separated by discontinuous Percoll density gradient centrifugation. The densities of the various layers of the Percoll were 1.107, 1.097, 1.092, 1.080 and 1.077. The mononuclear layer containing monocytes and lymphocytes, and neutrophil layer were isolated, washed with PIPES buffer and centrifuged. Monocytes were separated from lymphocytes by plastic adherence method. By this method, the purity of the WBCs was as follows: neutrophils >95%, monocytes >90%, and lymphocytes >92%. The viability of the cells, as measured by trypan blue dye exclusion, was greater than 98%.

Leukocyte Labeling: The isolated lymphocytes, monocytes, and neutrophils were labeled with 51-sodium chromate [^{51}Cr] (New England Nuclear, Boston, MA). The cells were suspended in phosphate-buffered saline (PBS) containing 5% glucose. Following quantitation of the cells using a Coulter counter, the leukocytes were incubated with 10 μCi of [^{51}Cr] per million cells in a 5% CO_2, 98% humidity incubator at 37°C for 60 minutes with constant agitation. The leukocytes were then washed three times with PBS containing 5% glucose and then resuspended in RPMI 1640 medium.

WBC Adherence Assay: HUVECs (passages 2-4) were grown to confluence in 96 well plates (no longer than 48 hr). At the start of the experiment, the media was changed from M-199 to RPMI 1640 without any FCS or growth factors. The HUVEC monolayers

were incubated (37°C, 5% CO_2, 98% humidity) with the tyrosine kinase inhibitors, genistein (30 μM) or herbimycin A (0.875 μM) for 10 min. Control wells of HUVECs were run concurrently containing medium only. The HUVECs were then treated with either tumor necrosis factor-α (TNF-α, 10 ng/ml) or interleukin-1 (IL-1, 10 ng/ml), and incubated for 2 hrs in the presence or absence of tyrosine kinase inhibitors. The media was then removed from the wells, and fresh RPMI 1640 media was added. The [^{51}Cr]-labeled leukocytes (100,000 cells/well) were distributed over the confluent HUVEC monolayers and co-incubated for 1 hr at 37°C. Following incubation, endothelial monolayers were gently washed three times with RPMI medium to remove non-adherent leukocytes. A 50 μl of fresh RPMI media was then added to each well. The HUVEC monolayers and adherent leukocytes were harvested using a cotton-tip applicator and swabbing the well. The radioactivity in the applicator tip was counted in a Beckman gamma counter with a window for ^{51}Cr.

Data Analysis: On each experimental day, assays were always run in triplicate for each group. Since all the WBCs were homogenously labeled with ^{51}Cr and there was no aggregation of the cells, the amount of the radioactivity was considered as equivalent to the density of the cells adhered to the HUVEC monolayer. In order to examine the change in the adherence of WBCs in response to TNFα or IL-1 in the presence or absence of tyrosine kinase inhibitors, the control basal adherence values were subtracted from the experimental group values. The percent inhibition of the adherence of WBCs by tyrosine kinase inhibitors was then calculated. Data has been analyzed by ANOVA, and the statistically significant difference was determined at 95% confidence limits ($p < 0.05$).

RESULTS

Basal Adhesion: The adherence of PMNs and lymphocytes in the absence of any stimulus was relatively constant under the conditions of the assay. An average of 12% of the incubated PMNs adhered to the HUVEC monolayers (range 6-21%, n=9), and an average of 13% of the incubated lymphocytes were adherent (range 8-25%, n=9). However, the basal percent adherence of monocytes was higher with an average of 47% (range 18-65%, n=6). The elevated adherence values seen with the monocytes may be due to the release of endogenous TNFα and IL-1 from the monocytes which may have been activated during the incubation.

Stimulated Adhesion: Both the cytokines, TNFα and IL-1, enhanced the adherence of neutrophils, lymphocytes, and monocytes in a time-dependent manner (data not shown). The maximum adherence was obtained at 2 hrs. Therefore, in the following experiments a two-hour incubation period with TNF-α or IL-1 was chosen to examine the effect of tyrosine kinase inhibitors. In the presence of IL-1 or TNF-α, the adherence of PMNs and lymphocytes increased 3-4 fold, whereas the increase in monocyte adherence was about 1.5-2 fold. Both in PMNs and lymphocytes, the effect of TNF-α was about 2-3 fold greater as compared with the effect of IL-1.

Effect of Tyrosine Kinase Inhibitors: The tyrosine kinase inhibitors, genistein and herbimycin-A, were effective in attenuating the adherence of WBCs (Fig. 1 & 2). In these studies, we have examined the effect of only one dose of the inhibitors, genistein 30 μM and herbimycin A 0.875 μM. At these doses these compounds completely and

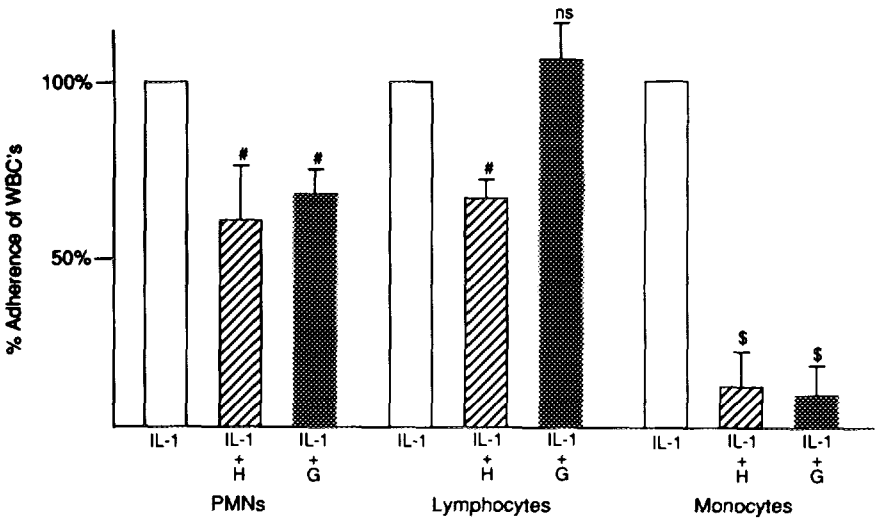

Fig. 1. Effect of tyrosine kinase inhibitors, herbimycin A (H) and genistein (G) on IL-1-stimulated human blood neutrophil (PMNs), lymphocyte, and monocyte adherence to HUVEC monolayers. Baseline adherence of WBCs was considered as zero percent, and increase in the adherence of WBCs induced by IL-1 was considered as 100%, as shown in open bars. Percent adherence of WBCs was calculated as mean ± SEM from 3-8 separate experiments. p values # <0.05, \$ <0.001 (as compared to IL-1 group); ns- not significant.

selectively inhibit tyrosine kinases in various cells without any effect on other kinases (6,7).

In case of neutrophils, both genistein and herbimycin A significantly inhibited ($p<0.05$) IL-1 or TNF-α-induced upregulation of cell adherence (Fig. 1 &2). However, the effect of genistein or herbimycin A was greater in the case of TNFα (maximum inhibition 47-57%; $p<0.01$) (Fig.2) as compared to that with IL-1 (maximum inhibition 31-37%; $p<0.05$) (Fig. 1). Herbimycin A inhibited IL-1-stimulated lymphocyte adherence to 68% ($p<0.05$). There was no effect of genistein on IL-1-induced lymphocyte adherence to HUVECs (Fig. 1). In fact, a slight potentiation was observed. In contrast, the TNF-α-induced lymphocyte adherence was significantly ($p<0.05$) inhibited by genistein and herbimycin-A (Fig. 2).

Both genistein and herbimycin A attenuated almost completely the IL-1-induced monocyte adherence (86-89%; $p<0.001$) (Fig. 1), whereas the TNFα-induced monocyte adherence was significantly inhibited (49-60%; $p<0.01$) (Fig. 2).

DISCUSSION

It has been well established that cytokines such as IL-1 and TNF-α enhance the expression of several cell adhesion molecules on the endothelium (8). The increased

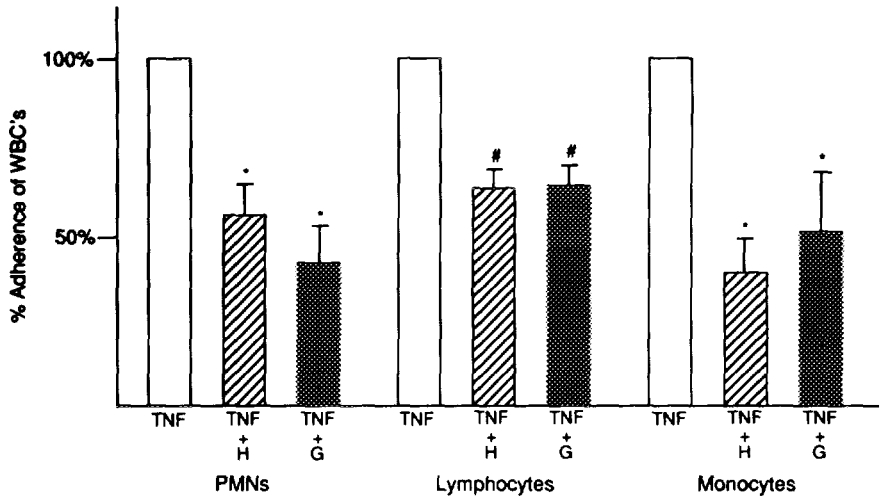

Fig. 2. Effect of tyrosine kinase inhibitors, herbimycin A (H) and genistein (G) on TNFα-stimulated human blood neutrophil (PMNs), lymphocyte, and monocyte adherence to HUVEC monolayers. Baseline adherence of WBCs was considered as zero percent, and increase in the adherence of WBCs induced by TNFα was considered as 100%, as shown in open bars. Percent adherence of WBCs was calculated as mean $\pm$ SEM from 3-8 separate experiments. p values # <0.05, * <0.01 (as compared to TNFα group); ns- not significant.

expression of cell adhesion molecules has been measured by investigating the adherence of white blood cells to the HUVECs. However, the mechanism(s) underlying such an increase in the expression of cell adhesion molecules is not well understood. In this study, we observed that two very potent and selective tyrosine kinase inhibitors, genistein and herbimycin A, attenuated the adherence of neutrophils, monocytes, and lymphocytes to HUVECs. This suggests that the tyrosine-phosphorylated proteins play an important role in the regulation of cell adhesion molecules on endothelial cells. However, the role of other proteins in regulating the transcription and translation of cell adhesion molecules cannot be ruled out.

In the present study, we observed that both genistein and herbimycin-A attenuated the adherence of neutrophils and monocytes in response to both IL-1 and TNF. However, IL-1-induced adherence of lymphocyte was not affected by genistein. In contrast, herbimycin A showed a significant inhibition. This suggests differential effects of genistein and herbimycin A on lymphocyte adherence which might involve different classes of cell adhesion molecules on the endothelium.

Interestingly, both genistein and herbimycin A were much more effective in inhibiting the monocyte adherence as compared to other cells; the monocyte adherence was almost completely abolished whereas neutrophil adherence was attenuated to about half. These data further support the possibility of the involvement of subtypes of cell

adhesion molecules in the adherence of monocytes and neutrophils. A number of cell-cell adhesion molecules have been recognized. These include vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1, and endothelial cell adhesion molecule-1 (8). It is possible that expression of the cell adhesion molecules could be differentially regulated by tyrosine phosphorylated proteins which have varing sensitivities to genistein and herbimycin A.

Vascular injury involving endothelial disruption following angioplasty or at sites of anastomoses initiates the margination and adherence of leukocytes to the endothelium (10). Endothelial cell activation or injury may thus initiate a series of processes that lead to hyperplasia and cholesteryl ester deposition in intimal smooth muscle cell and macrophages. Activated or regenerated endothelium may also promote the transition of smooth muscle cells from the contractile to secretory phenotype, promoting smooth muscle cell proliferation.

The tyrosine kinases are responsible for less than 1% of protein phosphorylation within cells. Tyrosine kinase activity has been observed to be associated with many growth factor receptors (such as EGF, PDGF, and insulin) and products of oncogenes such as src, fms, abl, fps, fes and ros (9). In studies related to cancer cell lines, investigators have found that dysregulation, leading to excessive signaling by receptor tyrosine kinases, results in cell proliferation that is inappropriate for nutritional and spatial conditions (9). The regulation of intimal repair following vascular injury may also be subject to such tyrosine kinase-dependent mechanisms, with the potential for excessive proliferation. Our data supports this possibility.

Our data suggest that tyrosine phosphorylation of certain proteins involved in the receptor activation of WBC mediators and/or of the cell adhesion molecules is an essential step in the interaction of leukocytes with endothelium. Further elucidation of the phosphorylated proteins involved will be helpful in the treatment and/or management of intimal hyperplasia and atherosclerosis.

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