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Effect of trapidil on effector functions of monocytes related to atherosclerotic plaque

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Abstract

The infiltration and activation of inflammatory cells play an important role in the formation and stability of coronary atherosclerotic plaque in patients with acute coronary syndrome. In this study, we evaluated the effect of trapidil, an anti-platelet agent, on atheroma-related functions of human T cells and monocytes. Trapidil and anti-CD154 (CD40 ligand) antibody inhibited the increase of procoagulant activity in the mixed lymphocyte reaction; trapidil also suppressed the induction of tissue factor, monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 in the mixed lymphocyte reaction. Trapidil did not alter CD154 expression on isolated T cells, but it diminished CD40 expression on isolated monocytes and human monocytic leukemia THP-1 cells stimulated with interferon-γ. Moreover, trapidil reduced MCP-1 production of isolated monocytes and THP-1 cells stimulated with interferon-γ plus CD154-transfected cells. This effect was not seen with other tested anti-platelet agents and coronary vasodilators. In conclusion, trapidil directly acts on monocytes/macrophages to lower their susceptibility to CD154 on T cells.

Keywords: Trapidil; Monocyte; T cell; Tissue factor; Matrix metalloproteinase; Monocyte chemoattractant protein-1 (MCP-1)

1. Introduction

Thrombosis, initiated by rupture or erosion of coronary atherosclerotic plaque, is the common cause of acute coronary syndromes such as unstable angina and myocardial infarction. Rupture-prone "vulnerable" plaques with a substantial lipid core are characterized by (1) the infiltration of large numbers of inflammatory cells including T cells and macrophages, and (2) a thin fibrous cap separating the thrombogenic macrophages from the blood (Fuster, 1994). The infiltration of inflammatory cells is mediated by chemokines, such as monocyte chemoattractant protein-1 (MCP-1) that attracts T cells and monocytes circulating in blood to recruit them into arterial sub-endothelium (Yla-Herttula et al., 1991). After migration, T cells trigger the inflammatory responses of atherosclerotic cells, including macrophages, through contact-dependent mediators such as CD154 (CD40 ligand) and humoral mediators such as interferon-γ (Mach et al., 1998; Libby, 1995). Infiltrated monocytes differentiate into macrophages. They express large amounts of tissue factor, a powerful procoagulant that potently stimulates thrombus formation, and release matrix metalloproteinases that weaken the fibrous cap (Libby, 1995).

Many in vitro studies have shown that the activation of human macrophages by CD154 on T cells induces tissue factor, matrix metalloproteinases and MCP-1 (Kuroiwa et al., 1999; Mach et al., 1997a; Malik et al., 1996). Interferon-γ from T cells may enhance these reactions because it up-regulates CD40, the counterpart of CD154 (Alderson et al., 1993). In fact, several reports have shown that the surface expression of CD154 on T cells increases (Aukrut et al., 1999) and serum tissue factor and MCP-1 levels are simultaneously elevated in patients with acute coronary syndromes (Nishiyama et al., 1998); other reports have shown that the expression of tissue factor (Moreno et al., 1996; Kaikita et al., 1997), CD40 (Mach et al., 1997b) and interferon-γ (Hansson et al., 1989) increases in human atherosclerotic lesions. In addition, in vivo studies using knock-out mice suggest that CD154, interferon-γ and MCP-1 are good targets for the treatment of atherosclerosis; the absence of CD154 (Lutgens et al., 1999), interferon-γ receptor (Gupta et al., 1997) or CC-chem-

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okine receptor-2, the receptor for MCP-1 (Bording et al., 1998), reduces the atherosclerotic lesion in apolipoprotein E knock-out mice. Thus, evidences above have revealed that these molecules play crucial roles in the pathogenesis of atherosclerosis.

Trapidil [7-diethylamino]-5-methyl-1,2,4-triazolo[1,5-a] pyrimidine] (Fig. 1) is clinically used as an anti-platelet agent or as a drug for angina pectoris. This drug has a complex spectrum of biological activities (Bönisch et al., 1998) including (1) inhibition of platelet function, (2) coronary vasodilation, and (3) inhibition of smooth muscle cell proliferation induced by platelet-derived growth factor. So far, a significant inhibition of restenosis after percutaneous trans-luminal coronary angioplasty has been reported in many clinical trials with trapidil, and its effect is explained by platelet-derived growth factor receptor antagonism (Nishikawa et al., 1992). A new aspect of the clinical use of trapidil has been demonstrated in the recent clinical trial named Japanese Antiplatelets Myocardial Infarction Study (JAMIS, Yasue et al., 1999), in which trapidil significantly reduced the incidence of cardiovascular events (reinfarction, unstable angina, etc.) after acute myocardial infarction. However, it is unlikely that this new clinical action of trapidil is caused merely by its known biological activities because of the following reasons. (1) The comparative anti-platelet agent used in the study, aspirin, was not so effective as trapidil. (2) The vasodilation induced by trapidil made a minor contribution because almost 80% of the patients had received nitrates and calcium antagonists. (3) The antimitogenic effect on smooth muscle cells might not have been beneficial for some patients with acute coronary syndromes who participated in the study, because smooth muscle cell proliferation in a fibrous cap is inhibited in vulnerable plaques. Surprisingly, a recent in vitro study has revealed that trapidil inhibits the CD40 pathway of monocytes, reducing the production of interleukin-6, interleukin-12 and procoagulant activity (Zhou et al., 1999). This new exciting finding in vitro and the above clinical observations led us to propose the hypothesis that trapidil might improve acute coronary syndromes by inhibiting interferon-γ- and CD154-mediated cellular functions directly related to the formation and/or stability of atherosclerotic plaques.

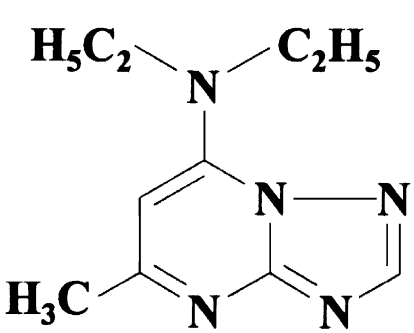

Fig. 1. Chemical structure of trapidil.

In the present study, we found an inhibitory effect of trapidil on MCP-1, matrix metalloproteinase-9 and tissue factor induction caused by the mixed lymphocyte reaction, a putative experimental model for the interaction between T cells and monocytes. Furthermore, we confirmed that the main target cell of trapidil was monocytes, using highly purified T cells/monocytes and human monocytic leukemia THP-1 cells. Finally, we tried to clarify the mechanism by which trapidil acts on monocytes, comparing the effects of various anti-platelet agents and coronary vasodilators with that of trapidil on the MCP-1 production by THP-1 stimulated with interferon-γ plus CD154.

2. Materials and methods

2.1. Chemical compounds

Trapidil (Rocornal®), cilostazol and nicorandil were prepared or extracted from corresponding tablets in our laboratory. Aspirin, cyclosporin A, dilazep, ticlopidine, isosorbide nitrate, polymixin B, phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma (St. Louis, MO). 1-methoxy-5-methyl phenazinium methylsulfate (1-methoxy PMS) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium sodium salt (WST-1) were purchased from Wako (Osaka, Japan).

2.2. Cytokines and antibodies

Interferon-γ was purchased from Genzyme-Techne (Cambridge, MA). Granulocyte–macrophage colony-stimulating factor (GM-CSF) was obtained from Pepro-Tech (London, England). Phycoerythrin-labeled and fluorescein isothiocyanate-labeled murine immunoglobulin (Ig) G1 isotypic control monoclonal antibodies (clone: 679.1 Mc7) were purchased from Immunotech (Cedex, France). Fluorescein isothiocyanate-labeled anti-tissue factor monoclonal antibody was purchased from American Diagnostica (Greenwich, CT). Phycoerythrin-labeled anti-CD14 monoclonal antibody (clone: TÜK4) was purchased from DAKO (Glostrup, Denmark). Phycoerythrin-labeled and unlabeled anti-CD154 blocking monoclonal antibody (clone: TRAP1), murine IgG1 isotypic control monoclonal antibody (clone: A112-2), phycoerythrin-labeled anti-CD54 (intercellular adhesion molecule-1: ICAM-1) monoclonal antibody (clone: HA58), phycoerythrin-labeled murine IgG2a isotypic control monoclonal antibody (G155-178), fluorescein isothiocyanate-labeled anti-CD4 monoclonal antibody (clone: RPA-T4) and anti-CD16/32 monoclonal antibody (Fc Block™, clone: 2.4G2) were purchased from Pharmingen (San Diego, CA).

2.3. Isolation of peripheral blood mononuclear cells, monocytes and helper T cells

Peripheral blood mononuclear cells of healthy volunteers were isolated from blood anti-coagulated by the

addition of sodium citrate at 0.4 w/v%. To avoid monocyte activation by a very low level of endotoxin (< 10 pg/ml), blood was collected in the presence of 10 μg/ml polymixin B, and all isolation procedures were carried out in the presence of 0.5 μg/ml polymixin B. After the density-gradient centrifugation of blood on a Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) at $400 \times g$ for 40 min, the obtained peripheral blood mononuclear cells were washed three times in Ca^{2+}/Mg^{2+}-free Hank's balanced saline solution (HBSS, Gibco Life Technologies, Paisley, Scotland) by centrifugation at $100 \times g$ for 10 min.

Monocytes were isolated from peripheral blood mononuclear cells by positive selection using anti-CD14 microbeads and a microbead-activated cell sorting system (MACS, Miltenyi Biotec, Gladbach, Germany). The obtained monocyte preparations contained more than 97% CD14-positive cells analyzed by flow cytometry, and were more than 97% monocytes under microscopic examination after Diff-Quick (Kokusai Shiyaku, Tokyo, Japan) staining.

Helper T cells were isolated from the CD14-negative fraction by CD4-positive selection in the microbead-activated cell sorter using anti-CD4 microbeads. The obtained helper T cell preparations contained more than 97% CD4-positive cells and were more than 97% lymphocytes.

The isolated cells were cultured in RPMI 1640 medium with 1 mM L-glutamine and 25 mM *N*-[2-hydroxymethyl] piperazine-*N*′-[2-ethanesulfinic acid] (HEPES, Gibco) supplemented with 50 μM 2-mercaptoethanol, 0.5 μg/ml polymixin B and 3% endotoxin-free decomplemented fetal calf serum (Gibco) at 37 °C in air supplemented with 5% CO_2.

2.4. Cell lines

Murine myeloma expressing human CD154 (CD154-transfected Ag8) and its parent cell, P3 × 63Ag8.628 (Ag8), were kindly donated by Dr. Takaomi Ishida. Human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Rockville, MD). They were cultured in RPMI 1640 medium supplemented with 1 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoethanol, 10% fetal calf serum and, sometimes, 10–50 μg/ml G-418 for CD154-transfected Ag8.

2.5. Mixed lymphocyte reaction

The mixed lymphocyte reaction was performed according to the method previously described (Pradier et al., 1996) with a slight modification. Briefly, peripheral blood mononuclear cells from unrelated donors (2×10^6 cells/ml) were mixed (allogeneic-mixed lymphocyte reaction (Allo)) or not mixed (autologous-mixed lymphocyte reaction (Auto)) and incubated for 18 h in the presence of vehicle (0.01% dimethyl sulfoxide: DMSO) or test compound. To study the effects of anti-CD154 antibody and corresponding isotypic control monoclonal antibody, peripheral blood mononuclear cells were pre-incubated for 30 min with these monoclonal antibodies before being mixed with lymphocytes. For the measurement of procoagulant activity, the cells in each well were washed twice with 50 mM Tris Buffer (pH 7.4) containing 150 mM NaCl and 0.1% bovine serum albumin. The washed cells at 100 μl/well were then frozen at −70 °C and stored until use. For enzyme-linked immunosolbent assay (ELISA) and flow-cytometric analysis, the supernatant was harvested and remaining cells were collected by gentle scraping.

2.6. The stimulation of isolated T cells and monocytes

Isolated T cells were seeded at 5×10^5 cells/ml and stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 6 h. THP-1 cells were seeded at 2×10^5 cells/ml and stimulated with interferon-γ (10 ng/ml) for 1 day. After incubation, the cells were collected by washing the plate with a buffer (HBSS containing 2 mM ethylenediamineteraacetic acid [EDTA] and 0.25% fetal calf serum) and analyzed by flow-cytometry. Isolated monocytes were seeded at 5×10^5 cells/ml and stimulated with interferon-γ (50 ng/ml) and GM-CSF (10 ng/ml) for 1 day. After incubation, monocytes were collected by gentle scraping and washed before analysis by flow-cytometry.

2.7. CD40 ligation by CD154-transfected Ag8

The priming and CD40 ligation of monocytes were performed according to the methods previously described (Pradier et al., 1996; Kuroiwa et al., 1999) with a slight modification. Isolated monocytes (1×10^6 cells/ml) were primed with interferon-γ and GM-CSF for 1 day. For the evaluation of surface tissue factor expression, monocytes were primed with interferon-γ (50 ng/ml) and GM-CSF (10 ng/ml). For the evaluation of cytokine production, they were primed with interferon-γ (10 ng/ml) and GM-CSF (1 ng/ml). After priming, CD154-transfected Ag8 or Ag8 was added at 1:1 ratio and co-cultured with the monocytes for 18 h. THP-1 cells were primed with interferon-γ (10 ng/ml), and further cultivation was performed after the addition of CD154-transfected Ag8 or Ag8 (1/4 or 1/10) for 18 h. Unless indicated, test compounds or vehicle (0.01% DMSO) was added to both the priming culture and the co-cultures to keep their concentrations unchanged.

2.8. Measurement of procoagulant activity

After thawing of the stored peripheral blood mononuclear cell samples, an equal volume of Tris buffer was added and the cell suspension was sonicated. The cell

homogenate was then mixed with an equal volume of 25 mM $CaCl_2$ solution (50 μl) and incubated for 2 min. Clotting time was recorded with an AMAX CS-190 fibrometer (MC Medical, Tokyo, Japan) after the addition of 50 μl of normal human plasma, and interpolated to the amount of standard thromboplastin (rabbit tissue factor: Simplastine, Organon Teknica, Durhan, NC).

2.9. ELISA and WST-1 assay

Total matrix metalloproteinase-9 activity (pro-matrix metalloproteinase-9 and active matrix metalloproteinase-9) in culture supernatant was measured using the BIOTRAK MMP-9 activity assay system (Amersham Pharmacia Biotech). For the measurement of MCP-1, 100 μl of culture supernatant was drawn from each well. The concentration of MCP-1 was determined using ELISA kits (A'NALIZA MCP-1, Genzyme-Techne). To evaluate the effects of test compounds on cell proliferation and viability, after sampling the culture supernatant, 10% (v/v) of WST-1 reagent (20 mM HEPES buffer pH 7.4, containing 5 mM WST-1 and 0.2 mM 1-methoxy-PMS) was added to each well. The culture was further incubated for 2 h followed by reading of the optimal density at 450 nm with a microplate reader.

2.10. Flow-cytometric analysis

The collected cells were incubated for 30 min at 4 °C with HBSS containing 2 mM EDTA, 5% mouse serum and anti-CD16/32 monoclonal antibody (10 μg/ml) to avoid non-specific binding of labeled monoclonal antibodies to human cells and Ag8. After this blocking step, cells were stained for 1 h with labeled monoclonal antibodies or corresponding labeled isotypic control monoclonal antibodies and then washed twice. Each sample was analyzed in a FACS Calibur Cytometer using CELL Quest software (Becton-Dickinson). The mean fluorescence intensity (MFI) was calculated as the geometric mean intensity and the value normalized to the log scale. The live T cells and monocytes were gated according to the sideward and forward light scatter properties. There were some technical difficulties in the measurement of tissue factor expression on monocytes co-cultured with CD154-transfected Ag8 or Ag8, because the murine myeloma has sideward and forward light scatter properties similar to those of monocytes, and anti-tissue factor monoclonal antibody cross-reacts to some extent with the cells. To overcome these difficulties, monocytes and THP-1 were double-stained with fluorescein isothiocyanate-labeled anti-tissue factor monoclonal antibody and phycoerythrin-labeled anti-intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody, and then tissue factor expression on live ICAM-1^{high} cells was specifically analyzed, because human monocytes changed into $CD14^{dim}$/ICAM-1^{high} during priming with interferon-γ, and anti-ICAM-1 monoclonal antibody did not cross-react with the murine cells.

2.11. Statistical analysis

Statistical analysis was done by using STAT LIGHT software 1997 (Yukms, Tokyo, Japan). A difference among groups was considered to be significant when $P < 0.05$. IC_{50} value with 95% reliable range was calculated using JMP® software 1995 (SAS Institute, Cary, NC).

3. Results

3.1. Effect of trapidil on atheroma-related parameters increased by mixed lymphocyte reaction

Fig. 2 shows the effects of two anti-platelet agents and anti-CD154 blocking monoclonal antibody on procoagulant activity induced by the allogeneic-mixed lymphocyte reaction. Anti-CD154 antibody at 10 μg/ml inhibited the induction of procoagulant activity but isotypic control antibody did not. As reported in a previous study (Zhou et al., 1999), trapidil at 10 to 100 μM inhibited the induction of procoagulant activity in a concentration-dependent manner. The activity obtained with the allogeneic-mixed lymphocyte reaction with trapidil at 100 μM was of the same magnitude as that obtained with the autologous-mixed lymphocyte reaction. In contrast, no inhibition was observed with aspirin at 100 μM.

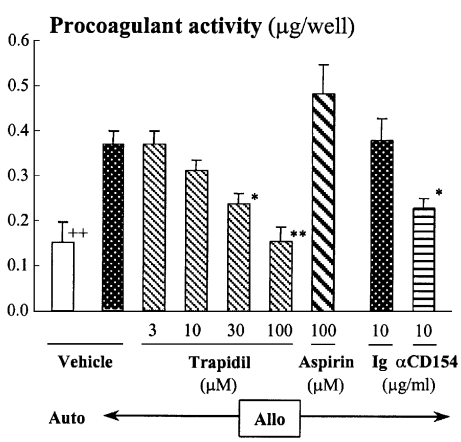

Fig. 2. Effect of trapidil and aspirin on procoagulant activity induced by the mixed lymphocyte reaction. Peripheral blood mononuclear cells were seeded in 96-well plates in autologous-mixed lymphocyte reaction (Auto) or allogeneic-mixed lymphocyte reaction (Allo) and then cultured in the presence of vehicle (0.01% DMSO) or test compounds. The neutralizing anti-CD154 monoclonal antibody (αCD154) and isotypic control monoclonal antibody (Ig) were simultaneously tested as reference. Each bar represents mean ± S.E.M. for cells from six different pairs of donors. $^{*}P < 0.05$ and $^{**}P < 0.01$ calculated by Dennett's test for multiple comparison vs. vehicle in Allo. $^{++}P < 0.01$ calculated by Student's *t*-test between Auto and vehicle in Allo.

To confirm whether the inhibitory effect of trapidil on procoagulant activity mirrors the reduction of tissue factor expression on monocytes, peripheral blood mononuclear cells were analyzed by flow-cytometry after double-staining with fluorescein isothiocyanate-labeled anti-tissue factor monoclonal antibody and phycoerythrin-labeled anti-CD14 monoclonal antibody. Almost all live monocytes, characterized by their sideward and forward light scatter properties, expressed CD14. Related to the change in procoagulant activity, we found that trapidil at 100 μM completely inhibited the increase in tissue factor expression on monocytes induced by the allogeneic-mixed lymphocyte reaction (Fig. 3, upper left panel). Simultaneously, the increase in CD40 expression on monocytes was significantly inhibited by trapidil at 100 μM (Fig. 3, upper right panel). To our surprise, the supernatant of the mixed lymphocyte culture contained detectable levels of MCP-1 and matrix metalloproteinase-9, and these levels were also increased by the allogeneic-mixed lymphocyte reaction. Trapidil at 100 μM suppressed the increase in MCP-1 and matrix metalloproteinase-9 levels, although the changes were not statistically significant (Fig. 3, lower panel).

3.2. Effect on CD154 expression on T cells and CD40 expression on monocytes

As anti-CD154 antibody was effective in inhibiting the procoagulant activity induced by the allogeneic-mixed lymphocyte reaction, CD154 on T cells and/or CD40 on monocytes were involved in the induction of procoagulant activity in the mixed lymphocyte reaction. However, it was difficult to determine in the experiment that either or both were the target cells of trapidil. To observe direct effects of drugs on individual cells, we purified helper T cells and monocytes using a microbead-activated cell sorter, and measured CD40/CD154 expression on these cells in response to stimuli (Table 1). The CD154 expression on resting T cells was very low, but the expression increased 20-fold in response to the addition of PMA plus ionomycin. As reported in a previous study (Fuleihan et al., 1994), cyclosporin A at 1 μM completely inhibited the induction of CD154 on T cells, whereas trapidil and aspirin at 100 μM did not. In contrast, trapidil significantly reduced CD40 expression on monocytes stimulated with interferon-γ plus GM-CSF by 27%.

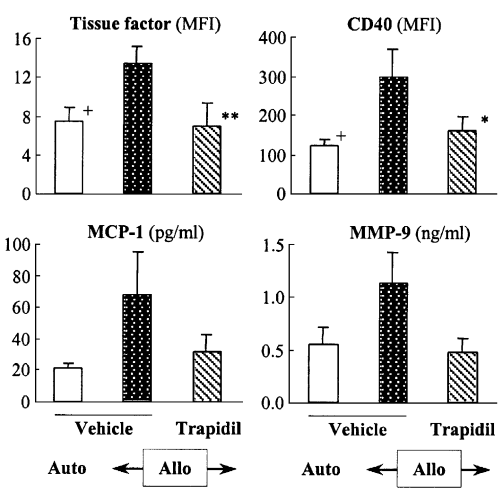

Fig. 3. Tissue factor and CD40 surface expression on monocytes, and MCP-1 and matrix metalloproteinase-9 production in culture supernatant after the mixed lymphocyte reaction. Peripheral blood mononuclear cells were mixed (Allo) or not mixed (Auto) with the peripheral blood mononuclear cells of unrelated donors and cultured in the presence of vehicle (0.01% DMSO) or trapidil (100 μM). After cultivation, the supernatant was harvested for ELISA, and the remaining peripheral blood mononuclear cells were stained with labeled-monoclonal antibody for flow cytometeric analysis to calculate mean fluorescence intensity (MFI). Each value represents mean ± S.E.M. for cells from four different pairs of donors. $^{*}P < 0.05$ and $^{**}P < 0.01$ calculated by paired *t*-test vs. vehicle (Allo). $^{+}P < 0.05$ calculated by Student's *t*-test between Auto and vehicle in Allo.

Table 1
Effect of drugs on CD154 expression on $CD4^+$ T cells and CD40 expression on $CD14^+$ monocytes

Stimuli	Treatment	CD154 on T cells (MFI)	CD40 on monocytes (MFI)
No	Vehicle	10 ± 1[a]	50 ± 6[a]
Yes	Vehicle	193 ± 40	714 ± 187
	Aspirin	164 ± 31	678 ± 165
	Trapidil	184 ± 31	528 ± 133[a]
	Cyclosporin A	14 ± 1[a]	NT

$CD4^+$ T cells and $CD14^+$ monocytes were isolated from human peripheral blood mononuclear cells by using a microbead-activated cell sorting system. T cells were stimulated with phorbol myristate acetate plus ionomycin for 6 h. Monocytes were stimulated with interferon-γ plus granulocyte–macrophage colony-stimulating factor. The cells were cultured in the presence of vehicle (0.01% DMSO), trapidil (100 μM), aspirin (100 μM) or cyclosporin A (1 μM). After stimulation, the cells were stained with labeled antibodies for flow cytometeric analysis to calculate mean fluorescence intensity (MFI). Results are expressed as means ± S.E.M. for four (T cells) or eight (monocytes) preparations from different donors.
NT: not tested.
[a] $P < 0.01$ vs. vehicle with stimulation by paired *t*-test.

3.3. Effect on tissue factor expression and MCP-1 production by isolated monocytes

Table 2 shows the effect of trapidil on tissue factor expression and MCP-1 production of human monocytes stimulated with CD154-transfected Ag8 after priming with interferon-γ plus GM-CSF. In our preliminary experiments, monocytes did not respond to CD40 ligation without priming with interferon-γ plus GM-CSF to up-regulate CD40 expression, as judged by MCP-1 production (data not shown). After priming, the co-culture with CD154-transfected Ag8 significantly increased tissue factor expression and the production of MCP-1 by monocytes when compared with those obtained by co-culture with the same number of Ag8. As shown in Table 2, aspirin at 100 μM

Table 2
Effect of drugs on tissue factor surface expression and MCP-1 production by monocytes stimulated with CD154 after the pre-incubation with IFN-γ and GM-CSF

Stimuli	Treatment	Tissue factor (MFI)	MCP-1 (pg/ml)
Ag8	Vehicle	30.6 ± 5.6[a]	350 ± 218[a]
CD154-Ag8	Vehicle	63.2 ± 14.4	1177 ± 485
	Aspirin	64.4 ± 12.5	988 ± 498
	Trapidil	52.8 ± 10.3[a]	248 ± 171[a]

$CD14^+$ monocytes were isolated from human peripheral blood mononuclear cells by using a microbead-activated cell sorting system. The monocytes were primed with interferon-γ (IFN-γ) plus granulocyte–macrophage colony-stimulating factor (GM-CSF) in the presence of vehicle (0.01% DMSO), trapidil (100 μM) or aspirin (100 μM). After priming, the monocytes were co-cultured with CD154-transfected Ag8 (CD154-Ag8) or Ag8 for further 18 h. The supernatant was harvested and MCP-1 level was analyzed by enzyme-linked immunosolbent assay. For flow cytometric analysis, monocytes were stained with labeled-monoclonal antibodies and then mean fluorescence intensity (MFI) was calculated. Results are expressed as means ± S.E.M. for cells from four different donors.

[a] $P < 0.05$ vs. vehicle with the stimulation with CD154-Ag8 by paired *t*-test.

did not affect these parameters. In contrast, trapidil significantly inhibited tissue factor induction and reduced MCP-1 production. Trapidil did not reduce ICAM-1 expression. The mean fluorescence intensity of ICAM-1 in vehicle-, aspirin- and trapidil-treated groups was 2357 ± 977, 2548 ± 968 and 2429 ± 975, respectively (mean ± S.E.M.). WST-1 assay, performed at the same time as the test for MCP-1 production, revealed that both drugs at 100 μM did not significantly affect cell viability. The optical density (at 450 nm) of vehicle-, aspirin- and trapidil-treated groups was 2.1 ± 0.1, 2.0 ± 0.0 and 2.0 ± 0.1, respectively (mean ± S.E.M.). Thus, the observed effects of trapidil could not be explained by its cytotoxic or anti-proliferative action. In an additional experiment, trapidil at 100 μM did not affect the surface CD154 expression on Ag8 (data not shown), indicating that the effect of trapidil was a direct action on monocytes and not on Ag8.

3.4. Effect of trapidil on THP-1

The amount of MCP-1 produced by isolated human monocytes varied among individual donors and different preparations, perhaps owing to their genetic backgrounds and physical conditions, which influenced the stability of the experiment. Therefore, we used another experimental system, using human monocytic leukemia THP-1 cells instead of isolated monocytes. As shown in Fig. 4A, THP-1 primed with interferon-γ exhibited the increase in CD40 expression. Trapidil at 100 μM slightly reduced the CD40 expression in the absence of interferon-γ (left panel) and abolished the up-regulation of CD40 in the presence of interferon-γ (right panel). Fig. 4B shows the atheroma-related function of THP-1 cells stimulated with CD154-

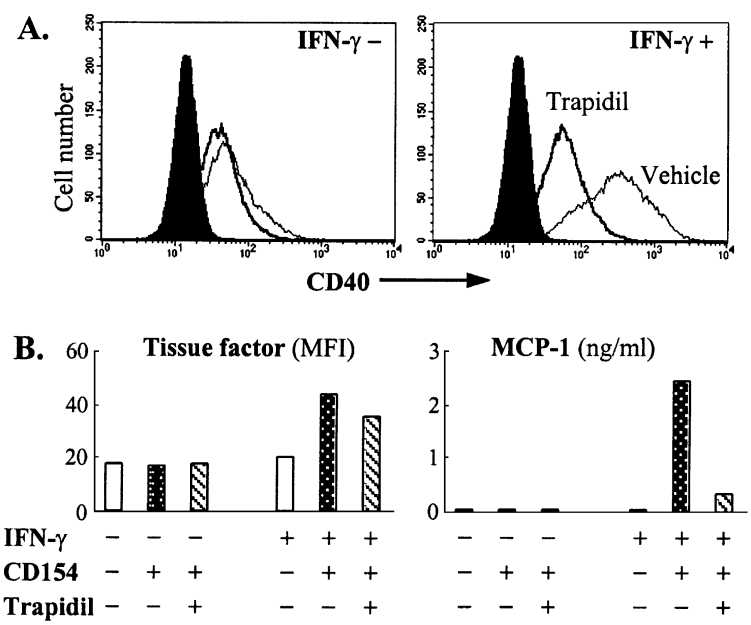

Fig. 4. Involvement of interferon-γ in the inhibitory effects of trapidil on CD40 expression and effector functions of THP-1. (A) CD40 expression on THP-1 in the presence (right panel) or absence (left panel) of interferon-γ (IFN-γ). Histograms with thin line and thick lines indicate the results of vehicle (0.01% DMSO) and trapidil (100 μM), respectively. The hatched histograms indicate staining with the isotypic control monoclonal antibody. (B) MCP-1 production and tissue factor induction in THP-1 elicited by CD40 ligation with or without interferon-γ priming. Open columns indicate co-culture with Ag8 cells (CD154 −). Hatched and gray columns indicate co-culture with CD154-transfected Ag8 cells in the absence (CD154 + , trapidil −) or presence (CD154 + , trapidil +) of trapidil at 100 μM, respectively. Each column represents the mean for four wells in one reproducible experiment.

transfected Ag8 after priming with or without interferon-γ. Only when THP-1 was primed with interferon-γ could the production of MCP-1 and the up-regulation of tissue factor be observed in response to CD40 ligation by CD154-transfected Ag8. Trapidil reduced the increase in these parameters induced by CD40 ligation after interferon-γ priming, and the most pronounced effect was observed on MCP-1 production. These data were similar to the results obtained in the experiment with human monocytes (Table 2).

3.5. Effect of trapidil added concomitantly to interferon-γ or CD154-transfected Ag8

Fig. 5 shows the effect of trapidil and anti-CD154 antibody on MCP-1 production in the experiment described above. Trapidil concomitantly added with interferon-γ reduced MCP-1 production in a concentration-dependent manner, whereas it was ineffective when added concomitantly with CD154-transfected Ag8. In contrast, the effect of anti-CD154 antibody was not so influenced by the timing of addition. On the basis of these data, apart from anti-CD154 antibody, trapidil was likely to act during priming with interferon-γ rather than during stimulation with CD154.

3.6. Comparison between the effect of trapidil and those of other compounds

Based on the concentration–response curve in Fig. 5, the IC_{50} value of trapidil was determined to be 13.6 μM

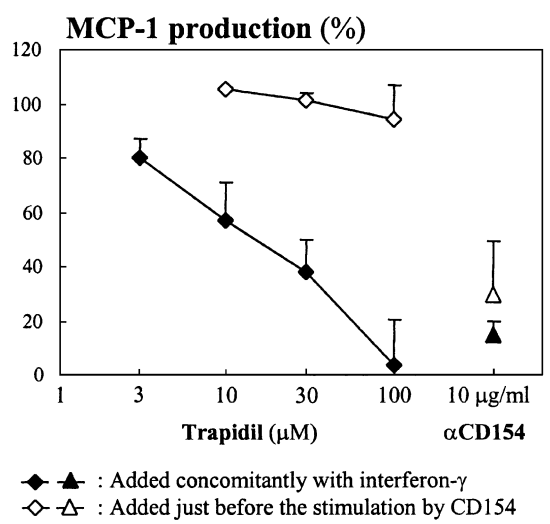

Fig. 5. Concentration–response curves for MCP-1 production by THP-1 stimulated with CD154 after priming with interferon-γ. MCP-1 levels are presented as percentage increase obtained by CD40 ligation relative to the levels observed with vehicle (for trapidil) and isotypic control monoclonal antibody (for anti-CD154 neutralizing monoclonal antibody: αCD154). Close symbols indicate the effects of trapidil or αCD154 added to both interferon-γ priming cultures and co-cultures with CD154-transfected cells to keep their concentrations unchanged. Open symbols indicate the effects of trapidil or αCD154 added only to the co-culture.

Table 3
IC_{50} values of anti-platelet agents and coronary vasodilators on MCP-1 production by THP-1, and their C_{max} and doses in clinical use

Drugs	IC_{50} (μM)	C_{max}[a] (μM)	Dose[b] (mg/body)
Trapidil	13.6	16.1	100
Aspirin	> 100	10.2	300
Ticlopidine	> 30	6.5	500
Cilostazol	> 10	2.1	100
Dilazep	> 10	0.2	100
Nicorandil	> 30	0.7	10
Isosorbide nitrate	> 30	0.02	20

THP-1 was primed with interferon-γ in the presence of vehicle (0.01% DMSO) or various drugs. After priming, THP-1 was co-cultured with CD154-transfected Ag8 or Ag8 for a further 18 h. The supernatant was harvested and the level of MCP-1 was analyzed by enzyme-linked immunosolbent assay. The cell viability was concomitantly monitored by WST-1 assay. Maximal concentration of each compound was determined by its solubility and its reduction of cell viability (not more than 20% reduction). IC_{50} values were calculated from concentration–response curves from at least three independent experiments.

[a]The maximal concentrations in plasma (C_{max}) and corresponding doses in clinical use are referred from the data of Japan Pharmaceutical Information Center.

[b]The maximal concentrations in plasma (C_{max}) and corresponding doses in clinical use are referred from the data of Japan Pharmaceutical Information Center.

(Table 3). The lower and upper limits of 95% reliable range were 5.6 and 26.1 μM, respectively. WST-1 assay, performed at the same time as the measurement of cytokine production, revealed that trapidil at the tested concentrations showed no effect on cell viability and proliferation. The IC_{50} value of trapidil was in the same range as the concentrations effective on MCP-1 production by monocytes and on procoagulant activity in the mixed lymphocyte reaction (Fig. 2), and was lower than the C_{max} value after administration of a therapeutic dose (Table 3). None of the tested anti-platelet agents (aspirin, cilostazol and ticlopidine) and coronary vasodilators (dilazep, isosorbide nitrates and nicorandil) inhibited MCP-1 production even at concentrations higher than the C_{max} values for their corresponding therapeutic doses.

4. Discussion

The effector functions of monocytes/macrophages related to the formation and stability of atherosclerotic plaques are considered to be induced by contact-dependent interaction with T cells, especially type 1 helper T cells (Th1) producing interferon-γ. CD40 ligation by CD154 on Th1 plays a pivotal role in the production of cytokines and chemokines and in the expression of tissue factor and matrix metalloproteinases by macrophages (Mach et al., 1998). Moreover, interferon-γ from Th1 may be one of the co-stimulatory signals which up-regulate CD40 expression on monocytes/macrophages (Alderson et al., 1993). The

mixed lymphocyte reaction is considered to be an experimental model of this Th1-monocyte interaction, in which Th1 is activated by allogeneic antigen on monocytes, resulting in the expression of CD154 and in the production of proinflammatory cytokines like interferon-γ; these molecules induce the effector functions of monocytes. Zhou et al. (1999) found that trapidil at therapeutic concentrations could inhibit the procoagulant activity induced by mixed lymphocyte reaction. In the present study, while reproducing their results on procoagulant activity, we found the following new findings: (1) the inhibitory effect of trapidil on procoagulant activity in the mixed lymphocyte reaction was accompanied by a reduction in the surface expression of tissue factor and CD40 on monocyte, at least at high dose (100 μM), and (2) trapidil could also inhibit matrix metalloproteinase-9 and MCP-1 production induced by the mixed lymphocyte reaction. The latter finding is of special interest because the subcutaneous administration of trapidil has been reported to inhibit MCP-1 production, macrophage accumulation and intimal hyperplasia after balloon arterial injury in hypercholesterolemic rabbits (Poon et al., 1999). This in vivo effect of trapidil may be explained by our in vitro finding that trapidil inhibited directly MCP-1 production induced by the Th1–monocyte interaction.

In addition, the inhibitory effects of trapidil on matrix metalloproteinase-9 release and on tissue factor induction may suggest other pharmacological properties useful for therapy aimed at controlling atherosclerotic plaques, because the down-regulation of matrix metalloproteinase-9 may reduce the breakdown of the fibrous cap, and the down-regulation of tissue factor may decrease the risk of thrombosis generation when plaques rupture. However, these possibilities should be confirmed by further in vivo studies using animal models of atherosclerosis, such as apolipoprotein E knock-out mouse or the hypercholesterolemic rabbits mentioned above.

In order to clarify the target cells of trapidil in the mixed lymphocyte reaction system, T cells and monocytes were highly purified and we investigated the effect of trapidil on CD154 expression on T cells and CD40 expression on monocytes stimulated with phorbol ester and interferon-γ plus GM-CSF, respectively. Trapidil reduced CD40 expression on monocytes, but it did not reduce CD154 expression on T cells. Therefore, monocytes are likely to be its main cellular target. This was also supported by the experimental result showing that trapidil inhibited MCP-1 production and tissue factor induction in isolated human monocytes and monocytic leukemia THP-1 co-cultured with Ag8 cells stably expressing CD154.

Concerning the mechanism by which trapidil inhibits cytokine production and procoagulant activity induction, a previous report suggested that trapidil may inhibit monocyte activation by reducing CD40 expression in isolated monocytes (Zhou et al., 1999). In the present study, confirming the inhibitory effect of trapidil on CD40 up-regulation in the mixed lymphocyte reaction, we also found a similar effect on monocytic leukemia THP-1 cells. Interestingly, we found that the addition of trapidil concomitantly with CD40 ligation could not inhibit MCP-1 production by THP-1 already primed with interferon-γ (Fig. 5, open squares). Moreover, the MCP-1 production of isolated monocytes or THP-1 was inhibited by low concentrations of trapidil (added concomitantly with interferon-γ) at which it could not significantly reduce CD40 expression (data not shown). These findings suggest that trapidil does not directly block the signal transduction pathway through CD40. The drug may inhibit the priming or differentiation of monocytes to desensitize their susceptibility to CD154 through the inhibition of co-stimulatory signals such as interferon-γ besides the reduction of CD40 expression at higher concentrations.

Trapidil is clinically used as an anti-platelet agent or a drug for angina pectoris. In addition to trapidil, there are many drugs available as anti-platelet agents and coronary vasodilators; therefore, we compared their effects on MCP-1 production with that of trapidil. As shown in Table 3, none of the tested anti-platelet agents (aspirin, ticlopidine, cilostazol and dilazep) and coronary vasodilators (nicorandil and isosorbide nitrate) showed any inhibitory activity at concentrations higher than their C_{max} values for therapeutic doses. Thus, the inhibitory effect on monocyte MCP-1 production as well as on monocyte priming mediated by interferon-γ is a unique pharmacological property of trapidil. These results also provide some considerations as to the molecular mechanism of trapidil: (1) nuclear factor κB and cyclooxygenase, known as the targets of aspirin, are unlikely to be involved, because aspirin did not affect CD40/tissue factor expression and the MCP-1 production of human monocytes or THP-1; (2) a K^+ channel opener, nicorandil, and a nitric oxide donor, isosorbide nitrate, were ineffective on THP-1, indicating that these molecules are not involved; (3) adenosine and intracellular cyclic AMP accumulation seemed not to be involved, because they are the targets of dilazep and cilostazol. Furthermore, the last mechanism is not involved for the following reasons: we confirmed that trapidil did not show any binding to adenosine receptors and purine receptors at 100 μM (data not shown); it has been reported that neither direct stimulation of cyclic AMP formation nor inhibition of phosphodiesterase is observed with therapeutic concentrations of trapidil (Bönisch et al., 1998). Since Bönisch et al. (1998) have suggested that trapidil inhibits smooth muscle cell proliferation and the kinase cascade through its direct activation of protein kinase A, monocyte/THP-1 priming by interferon-γ may be inhibited by the same mechanism. However, this possibility should be confirmed by further studies using the THP-1 model used in this study.

In summary, trapidil directly acted on monocytes to prevent MCP-1 production in response to CD40 ligation. Since this pharmacological property was unique to trapidil

and observed at clinically used concentrations, the clinical finding that trapidil prevents the incidence of acute coronary syndromes may be explained by, at least partially, its inhibitory effect on the atherosclerosis-related effector function of monocytes. A future investigation regarding the molecular mechanism of trapidil may provide insight into new targets for therapy aimed at controlling the formation and stability of atherosclerotic plaques.

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