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Effect of tranilast on matrix metalloproteinase production from neutrophils in-vitro

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Abstract

Tranilast is an anti-allergic agent that blocks the release of chemical mediators, such as histamine and leukotrienes from mast cells, and has been reported to suppress keloid and hypertrophic scar formation. Since matrix metalloproteinases (MMPs) play an essential role in tissue remodelling, this study was undertaken to determine whether tranilast suppresses MMP production from neutrophils after lipopolysaccharide (LPS) stimulation in-vitro. Neutrophils from five healthy donors (1×10^5 cells/mL) were stimulated with 1.0 μg mL^{-1} LPS in the presence or absence of various concentrations of tranilast for 24 h. MMP-7, MMP-8, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 levels in the culture supernatants were assayed by ELISA. In addition, the influence of tranilast on MMP mRNA expression and transcriptional factor activation in cells cultured for 12 h and 4 h was also evaluated by reverse transcriptase–polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Tranilast inhibited MMP and TIMP-1 production from neutrophils when cells were treated with the agent at more than 5.0×10^{-5} M. It also suppressed MMP mRNA expression and transcriptional factor activation induced in neutrophils by LPS stimulation. The results suggest that tranilast inhibits the formation of keloid scarring through the suppression of factors such as MMPs and TIMP, which are essential for tissue remodelling, from inflammatory cells.

Introduction

Tranilast (*N*-(3,4-dimethoxycinnamoyl) anthranilic acid) is used clinically in Japan for the treatment of allergic disorders, such as atopic dermatitis, bronchial asthma and pollinosis, with remarkable success (Azuma et al 1976; Ukai et al 1993). The clinical efficacy of tranilast has been found to depend on inhibition of the release of chemical mediators and inhibition of hypersensitivity reactions (Azuma et al 1976; Isaji et al 1997). In addition to its anti-allergic effect, several studies have shown that tranilast can prevent, or act to improve, keloid and hypertrophic scars (Isaji et al 1994; Suzawa et al 1992a). Although it has been speculated that these inhibitory effects of tranilast are, in part, attributable to its suppressive action on proliferation and collagen biosynthesis by fibroblasts (Isaji et al 1994, 1997; Suzawa et al 1992a), which are responsible for keloid formation and hypertrophic scars, the precise mechanisms are not well understood (Maita et al 2004).

The process by which tissue repair takes place is referred to as the wound healing period, and it comprises a continuous sequence of inflammation and repair, in which several types of cells (e.g., epithelial cells, inflammatory cells, fibroblasts, etc.) briefly come together outside their normal domains, interact to restore a semblance of their usual discipline and having done so resume their normal function. Wound healing has been arbitrarily divided into three phases: inflammation, proliferation and maturation (Redd et al 2004; Richardson 2004). Within 6 h after an injury, circulating immune cells start to appear in the wound. Polymorphonuclear leucocytes, especially neutrophils, are the first blood leucocytes to enter the wound site. Their numbers increase steadily and peak at 24–48 h. In the absence of infection at the wound site, their numbers decrease rapidly after the third day. The next cellular immune element to enter the wound is macrophages. These cells first appear within 48–96 h post-injury, and their numbers reach a peak at about the third day. These macrophages have a longer life span than other inflammatory cells, and they persist in the wound until healing is complete. Their appearance is followed by T cells, which appear in significant numbers at

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about the fifth to the seventh day post-injury. The recruitment of circulating immune cells into inflammatory sites involves traversing both the postcapillary venule walls and the interstitium. To traverse these barriers, immune cells adhere to endothelial cells and degrade extracellular matrix proteins (Hashimoto et al 2001; Kanai et al 2004a). The degradation of extracellular matrix proteins, including basement membrane proteins, is regulated by matrix metalloproteinases (MMP) and their inhibitors (tissue inhibitors of metalloproteinase; TIMP), which are secreted by a wide variety of cells, including immune cells (Hashimoto et al 2001; Kanai et al 2004a). Previous reports clearly show the suppression of MMP (MMP-1, -2 and -9) production from both gingival (Maita et al 2004) and nasal fibroblasts (Shimizu et al 2005) by tranilast, suggesting that this suppressive activity may contribute to inhibition of keloid formation and hypertrophic scars. However, the influence of tranilast on MMP production from inflammatory immune cells, such as macrophages and neutrophils, which are important for wound healing, is not well defined. This study, therefore, was undertaken to investigate the effect of tranilast on MMP production from neutrophils in response to lipopolysaccharide (LPS) stimulation in-vitro.

Materials and Methods

Agents

Tranilast (*N*-(3,4-dimethoxycinnamoyl) anthranilic acid) was kindly donated by Kissei Pharmaceutical Co. Ltd (Matsumoto, Japan) as a preservative-free pure powder. This was dissolved in antibiotic-free RPMI-1640 medium (Sigma Chemicals, Co. Ltd, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (RPMI-FCS; Flow Laboratories, North Ride, Australia) at 10^{-2} M. This solution was sterilized by passing through a 0.22-μm filter and stored at 4°C as a stock solution. All dilutions of tranilast used in this study were prepared from this stock solution. LPS (Sigma Chemical Co. Ltd) was dissolved in RPMI-FCS at 2.0 μg mL^{-1}. Monoclonal antibodies against human MMP-7, -8 and -9 were purchased from R & D Systems Inc. (Minneapolis, MN, USA). LPS extracted from *Klebsiella pneumoniae* was purchased from Sigma Chemical Co. Ltd and dissolved in RPMI-FCS. Basement membrane matrix (Matrigel) was purchased from Becton Dickinson Labware (Bedford, MA, USA).

Cell preparation

Heparinized human venous blood was obtained from five healthy subjects (all male, 25–52 years) after obtaining their written informed consent. The blood was diluted twice with phosphate-buffered saline (PBS) and layered onto Mon-Poly Resolving Medium (Flow Laboratories, Inc., MacLean, VA, USA). After centrifugation at 1000 *g* for 30 min at 25°C, peripheral blood leucocytes (PBLs) were collected from the plasma–medium interface and washed three times with PBS. CD16$^+$ cells, neutrophils, were separated from PBL using a magnetic cell separator (Milteny Biotec, Bergisch Gladbach, Germany). PBLs were labelled with human CD16 monoclonal antibody-coated magnetic beads (Milteny Biotec) and then applied to a column placed in the separator. After removing unlabelled cells by washing with PBS, the column was removed from the separator and rinsed with PBS. The entire eluted cells were washed twice with RPMI-FCS, resuspended at a concentration of 1×10^6 cells/mL, and used as neutrophils. The purity of neutrophils was more than 97% as judged by Giemsa's stain.

Cell culture

Neutrophils (1×10^5 cells/mL) were introduced into each well of 24-well plates, which contained 1.0 μg mL^{-1} LPS and various concentrations of tranilast in a final volume of 2.0 mL. The plates were then maintained at 37°C in a humidified atmosphere with 5% CO_2. The supernatant was collected 24 h later and stored at −40°C until assayed for the levels of both MMP and TIMP. To prepare cells to be examined for transcription factor activity and mRNA expression, neutrophils were cultured in a similar manner for 4 h and 12 h, respectively. In all experiments, tranilast treatment was started 1 h before LPS stimulation.

Transmigration assay

Neutrophil migration activity was assessed by Transwell plates as described previously with some modifications (Hashimoto et al 2001; Kanai et al 2004b). Transwell chambers fitted with polyvinylpyrolidine-free polycarbonate filters with 3.0-μm pore size were used (Corning Coster Corp., Cambridge, MA, USA). Each filter was coated with 50 μL of Matrigel (200 μg) in RPMI-1640 medium without FCS and incubated for 1 h at 37°C to create a thin continuous barrier on the top of the filter. To assess the influence of MMP-7, -8 and -9 on neutrophil migration, cells were seeded onto the coated membrane in the upper chamber of the Transwell at 1×10^6 cells in 500 μL of RPMI-FCS, and stimulated with 1.0 μg mL^{-1} LPS in the absence or presence of antibodies to MMP-7, −8 and −9 (5.0 μg mL^{-1} each). The lower chamber was filled with 1.0 mL of RPMI-FCS containing the same concentration of both LPS and monoclonal antibodies as the upper chamber. The cells were then cultured for 24 h, and the medium was harvested from the lower chamber. The number of cells in the medium was counted under a microscope. The number of cells that had adhered to the bottom of the lower chamber and the lower surface of membrane was also counted. To examine the influence of tranilast on neutrophil migration, cells (1×10^6) seeded onto a Matrigel-uncoated membrane in the upper chamber were cultured in the presence of various concentrations of tranilast in a total volume of 500 μL of RPMI-FCS. The lower chamber was filled with 1.0 mL of RPMI-FCS containing the same concentration of tranilast as the corresponding culture wells (the upper chamber) and cells that had migrated after 24 h were counted in a similar manner. The results were expressed as % of migration calculated as follows:

$$(\text{number of cells migrated}/1\times10^6 \text{ cells})\times100.$$

Assay for MMPs and TIMP-1

MMP and TIMP-1 levels in culture supernatants were measured in duplicate with commercially available MMP-7,

MMP-8, MMP-9 and TIMP-1 ELISA test kits (Amersham Biosciences Corp., Piscataway, NJ, USA) according to the manufacturer's recommendation. The sensitivity of the ELISA kits for MMP-7, MMP-8, MMP-9 and TIMP-1 was 0.16 ng mL^{-1}, 1.7 ng mL^{-1}, 0.6 ng mL^{-1} and 3.0 ng mL^{-1}, respectively.

Assay for mRNA expression

mRNA expression was assessed by means of the reverse transcriptase–polymerase chain reaction (RT-PCR). Poly A$^+$ mRNA was extracted from neutrophils with μMACS mRNA isolation kits (Milteny Biotec) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1.0 μg mRNA with a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA). Amplification of cDNA (1.0 μL) was performed with a Takara PCR Amplification kit (Takara Shuzo, Co. Ltd., Shiga, Japan) using specific primers for MMP and β-actin in a final volume of 30 μL. The primers used for RT-PCR were 5′-AGCCAAACTCAAGGAGATGC-3′ (sense) and 5′-ACTCCACATCTGGGCTTCTG-3′ (antisense) for MMP-7 (Barille et al 1999), 5′-ATGGCACAACACCTC CGCAA-3′ (sense) and 5′-GTCAATTGCTTGGACGCTGC-3′ (antisense) for MMP-8 (Arechavaleta-Velasco et al 2004), 5′-CCCACATTTGACGTCCAGAGAAGAA-3′ (sense) and 5′-GTTTTTGATGCTATTGGCTGAGATCCA-3′ (anti-sense) for MMP-9 and 5′-CGGAACCGCTCATTGCC-3′ (sense) and 5′-ACCCACACTGTGCCCATCTA-3′ (antisense) for β-actin (Kanai et al 2004b). The PCR conditions for MMP-9 and β-actin were: 4 min at 94°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C and 30 s at 70°C (Kanai et al 2004b). The conditions for MMP-7 (Barille et al 1999) were: 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 56°C and 60 s at 72°C, and for MMP-8 (Arechavaleta-Velasco et al 2004) 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 45 s at 72°C. After cycling, there was a DNA extension period of 2 min at 70°C (Barille et al 1999; Arechavaleta-Velasco et al 2004; Kanai et al 2004b). Each PCR product (10 μL) was run on 3% agarose gels, visualized with a UV illuminator after SYBR Green (BioWhittaker Molecular Applications, Rockland, ME, USA) staining and photographed. Intensity of mRNA level was corrected by β-actin transcripts calculated by a densitometer.

Assay for nuclear factor-κB (NF-κB) and transcription factor AP-1 activity

NF-κB activity was analysed with a commercially available ELISA test kit (Active Motif, Carlsbad, CA, USA) containing sufficient reagents and monoclonal antibodies against P50 and P65 by following the manufacturer's recommended procedure. In brief, nuclear extract (5.0 μg protein) from neutrophils was introduced into each well of 96-well microtitre plates pre-coated with oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) in a volume of 20.0 μL, and incubated for 1 h at 25°C. After washing three times, 100 μL of monoclonal antibody to P50 or P65 was added to the appropriate wells and incubated for a further 1 h at 25°C. Anti-IgG HRP-conjugate in a volume of 100 μL was then added and incubation was continued for another 1 h at 25°C. Absorbance at 450 nm was measured after the addition of tetramethylbenzine solution. AP-1 activity was also assessed in a similar manner by using a commercially available ELISA test kit (Active Motif) that contains sufficient reagents and monoclonal antibodies to Fra 1 and Jun B.

Statistical analysis

The statistical significance of the difference between the control and experimental data was analysed using analysis of variance followed by Fisher's PLSD test. $P < 0.05$ was considered significant.

Results

Influence of tranilast on neutrophil migration

The first set of experiments was undertaken to determine whether neutrophils are capable of degrading Matrigel, which resembles basement membrane, and migrate through it, and to determine whether tranilast is capable of inhibiting neutrophil migration. The influence of tranilast on neutrophil migration was first examined with a transwell filter that was not coated with Matrigel. Tranilast did not prevent spontaneous neutrophil migration even when the cells were treated with a concentration of 5.0×10^{-5} M (Figure 1A). We then examined the influence of tranilast on neutrophil migration through a Matrigel transwell filter, when the cells were stimulated with LPS in the presence of various concentrations of tranilast. LPS stimulation caused a significant increase in migration of neutrophils through the Matrigel transwell filter (Figure 1B; LPS alone), and this effect was significantly inhibited by the addition of anti-MMP to the culture medium (Figure 1B; LPS + anti-MMP). The data in Figure 1B clearly show that neutrophil migration, which was induced by LPS stimulation, was suppressed by the addition of tranilast into the culture medium and that the minimum concentration of tranilast that caused significant suppression was 5.0×10^{-5} M.

Influence of tranilast on MMP and TIMP-1 production from neutrophils in response to LPS stimulation in-vitro

The second set of experiments was undertaken to assess the effect of tranilast on MMP and TIMP-1 production by neutrophils in response to LPS stimulation in-vitro. Neutrophils were stimulated with 1.0 μg mL^{-1} LPS for 24 h in the presence or absence of various concentrations of tranilast. The MMP and TIMP-1 levels were analysed by ELISA. Lower concentrations of tranilast ($0.5–2.5 \times 10^{-5}$ M) did not suppress MMP-7 production from neutrophils, which was increased by LPS stimulation (Figure 2A): experimental culture supernatants contained nearly identical (not significant) levels of MMP-7 to that in control supernatants collected from cells stimulated with LPS alone. However, tranilast at a concentration of 5.0×10^{-5} M and higher caused significant suppression of MMP-7 production. We then investigated whether tranilast is also capable of suppressing the ability of neutrophils to produce MMP-8 in response to LPS stimulation. The data in

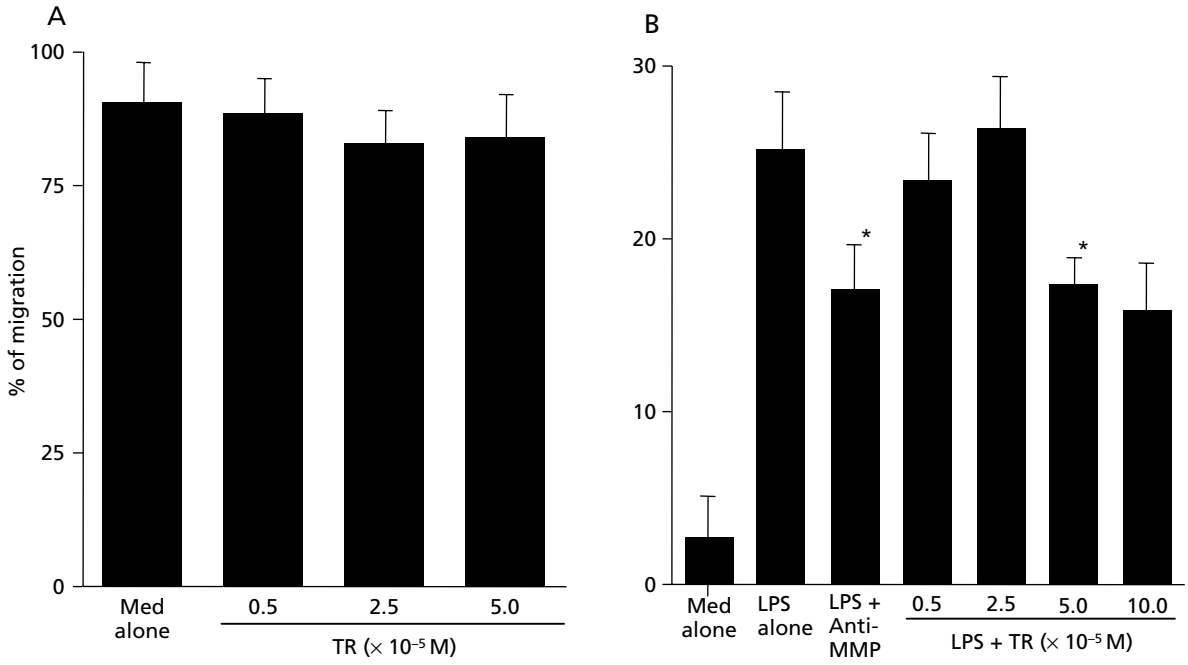

Figure 1 Influence of tranilast (TR) on neutrophil migration assessed by transmigration assay. A. Neutrophils (1×10^6 cells/500 μL) were cultured on a Matrigel-uncoated transwell filter. B. Neutrophils (1×10^6 cells/500 μL) were stimulated with 1.0 μg mL^{-1} LPS in the presence of various concentrations of tranilast on a Matrigel-coated transwell filter. After 24 h, the numbers of cells transmigrated were counted. The data are expressed as mean percent neutrophil migration ± s.e.m. of five different subjects. *$P < 0.05$, vs LPS alone.

Figure 2B clearly show that tranilast could suppress the production of MMP-8 from neutrophils when the cells were stimulated with LPS in the presence of tranilast at more than 5.0×10^{-5} M. Tranilast suppressed MMP-9 production as in the case of MMP-8 (Figure 2C). Since MMP production from cells in response to several types of stimulation has been reported to be associated with production of TIMP, we investigated the influence of tranilast on TIMP production from neutrophils in response to LPS stimulation. The data in Figure 2D clearly show that tranilast suppressed TIMP-1 production from neutrophils stimulated by LPS. The minimum concentration of tranilast that caused significant suppression of TIMP-1 production was 5.0×10^{-5} M.

Influence of tranilast on mRNA expression for MMP and TIMP-1 in neutrophils

The third set of experiments was carried out to examine whether tranilast could suppress mRNA expression for MMP and TIMP-1 and result in protein production. As shown in photographs in Figure 3, the addition of tranilast into the cell culture caused significant suppression of the mRNA expression examined. This suppression by tranilast was confirmed by graphs showing the ratios of target/β-actin.

Influence of tranilast on transcriptional factor activation in neutrophils

The final set of experiments was designed to examine the possible mechanisms by which tranilast suppresses MMP and TIMP-1 mRNA expression. To do this, we examined transcriptional factor, NF-κB and AP-1, activation induced by LPS stimulation. As shown in Figure 4, tranilast suppressed LPS-stimulated NF-κB activation. This was dose dependent, and the minimum concentration of tranilast for suppression of P50 and P65 was 5.0×10^{-5} M and 2.5×10^{-5} M, respectively. The data in Figure 5 also show the suppressive activity of tranilast at 5.0×10^{-5} M on AP-1 (Jun B and Fra-1) activation enhanced by LPS stimulation.

Discussion

The wound inflammatory response, the so-called inflammatory phase, can be divided into early inflammation, in which neutrophil infiltration predominates, and late inflammation, which is characterized by a mononuclear cell infiltration consisting of macrophages and lymphocytes (Redd et al 2004; Richardson 2004). After the clearing of devitalized and unwanted materials, including macroorganisms, at inflammatory phase it gives way to the proliferative phase of healing characterized by the formation of granulation tissue in the wound (Redd et al 2004; Richardson 2004). Granulation tissue consists of a combination of cellular elements, including fibroblasts and inflammatory cells, along with new capillaries embedded in a loose extracellular matrix of collagen, fibronectin and hyaluronic acid (Redd et al 2004; Richardson 2004). These morphological changes are now called tissue remodelling and are important in the development of inflammatory diseases, such as arthritis and bronchial asthma, as well as wound repair (Hashimoto et al 2001; Salib & Howarth 2003; Kanai et al 2004a). Tissue remodelling has also been linked to alterations in the activity of both MMPs and TIMP. The

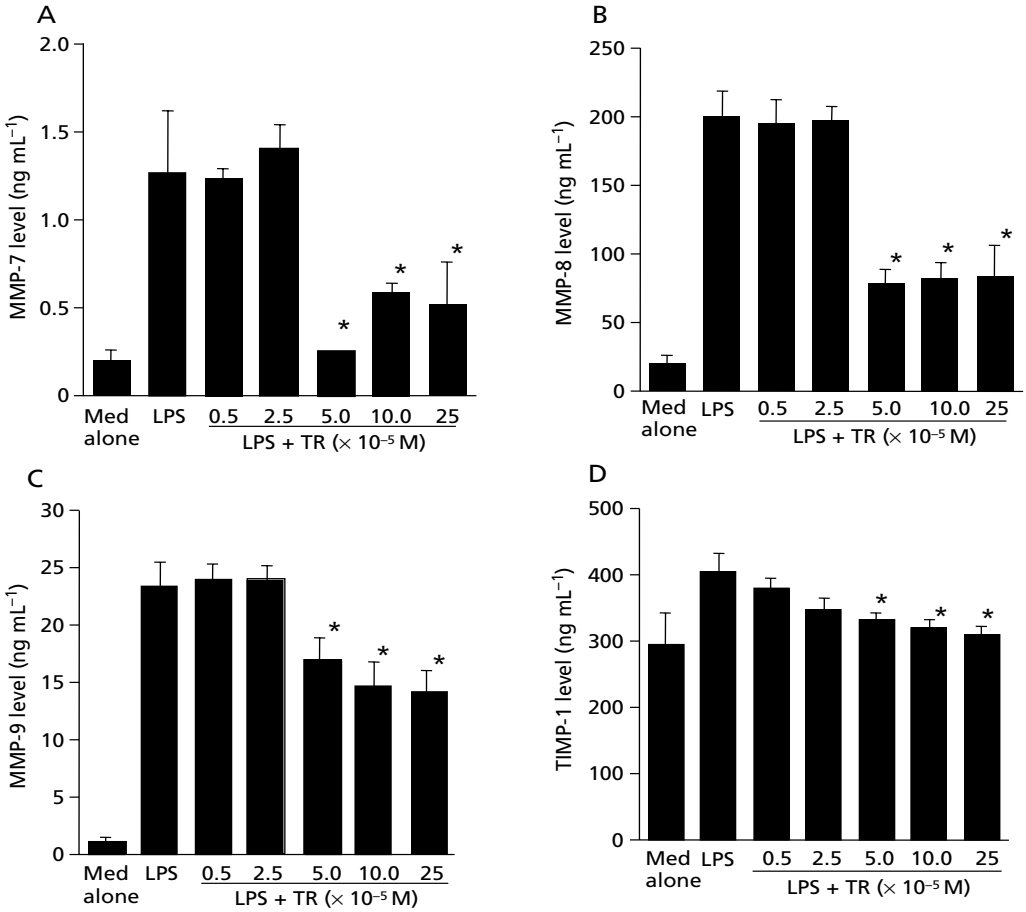

Figure 2 Influence of tranilast (TR) on the production of MMP and TIMP from neutrophils in response to LPS stimulation. Neutrophils prepared from human peripheral blood (1×10^5 cells/mL) were stimulated with 1.0 μg mL^{-1} LPS in the presence of various concentrations of tranilast for 24 h. Matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) levels in the culture supernatants were assayed by ELISA. The data are expressed as mean (ng mL^{-1}) ± s.e.m. of five different subjects. A. MMP-7. B. MMP-8. C. MMP-9. D. TIMP-1. *$P < 0.05$, vs LPS alone.

MMPs are a family of Ca^{2+}-activated, Zn^{2+}-dependent endopeptidases that can degrade various components of the extracellular matrix and basement membrane (Birkedal-Hansen 1995). More than 20 members of MMP have been identified to date, and they have been classified into several groups based on their substrate specificity and structure (Nagase & Woessner 1999). MMP-8 is secreted mainly by neutrophils and preferentially degrades type I, II, III and X collagen (Arechavaleta-Velasco et al 2004), whereas MMP-9 specifically degrades type IV and V collagen, denatured collagen and elastin (Hashimoto et al 2001; Kanai et al 2004a, b), which are the most important components of the extracellular matrix and basement membrane. MMP may also be implicated in microvascular permeability, leading to oedema and enhancement of cell migration (Ohno et al 1997; Hoshino et al 1998). Taken together, our results may be interpreted that the suppressive effect of tranilast on MMP production at 5.0×10^{-5} M, which is lower than therapeutic blood levels (3×10^{-4} M) (Suzawa et al 1992b), constitutes the therapeutic mode of action of tranilast on inflammatory diseases, including wound repair.

Most MMPs are secreted in an inactive proenzyme form and are subsequently activated in the pericellular and extracellular environment by plasminogen and elastase, among others (Birkedal-Hansen 1995; Nagase & Woessner 1999). Secreted pro-MMP is also activated by various proteinases belonging to the MMP family: membrane-type MMPs (MT-MMPs) can activate pro-MMP-2, and MMP-7 has been described as a potent MMP-1 and -9 activator (Birkedal-Hansen 1995; Nagase & Woessner 1999). From these reports, it is reasonable to speculate that the suppressive action of tranilast on MMP-7 production is partially responsible for the attenuating effect of tranilast on the development of remodelling in inflammatory tissues. In contrast to the MMP-inhibitory action of TIMPs, they have been shown to have a mitogenic action on a number of cell types and to enhance cells growth (Gomez et al 1997). TIMPs also exert an inhibitory effect on the growth of fibroblasts (Herbst et al 1997; Kim et al 2005), which are the most important component of granuloma observed in inflammatory sites. In addition to these TIMP functions as a controller of cell growth, TIMPs inhibit TNF-α-converting enzyme activation and IL-6 receptor shedding (Amour et al 1998), suggesting that the suppressive activity of tranilast on TIMP-1 production may be partially responsible for its anti-inflammatory action and inhibition of hypertrophic scarring and keloid formation.

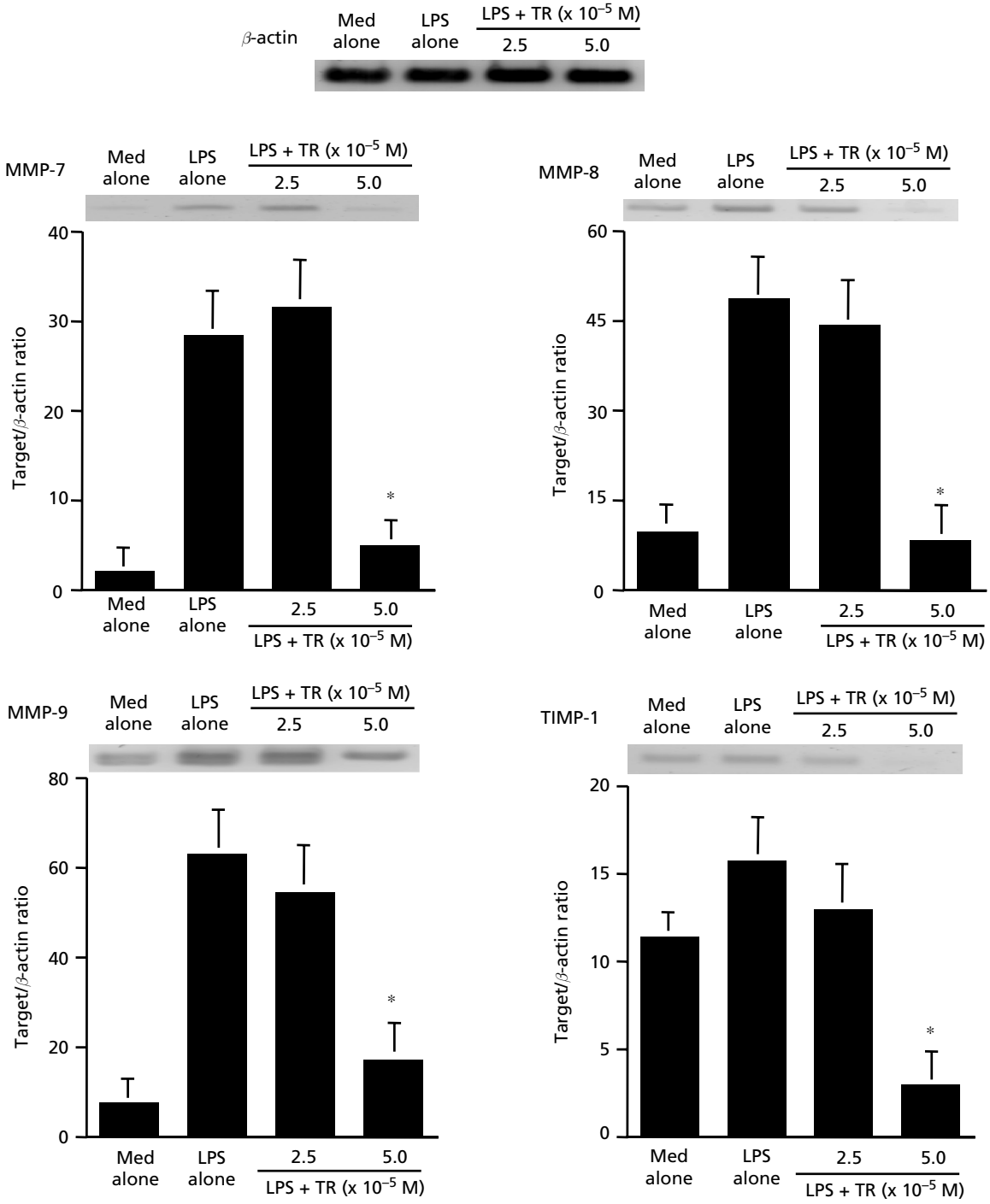

Figure 3 Influence of tranilast (TR) on MMP and TIMP mRNA expression in neutrophils stimulated with LPS. Neutrophils prepared from human peripheral blood (1×10^5 cells/mL) were stimulated with 1.0 μg mL^{-1} LPS in the presence of various concentrations of tranilast for 12 h. mRNA expression was assessed by RT-PCR. Photographs are one typical result out of five different subjects. Graphs show the ratio of MMPs/β-actin and are expressed as mean ± s.e.m. of five different subjects. *$P < 0.05$, vs LPS alone.

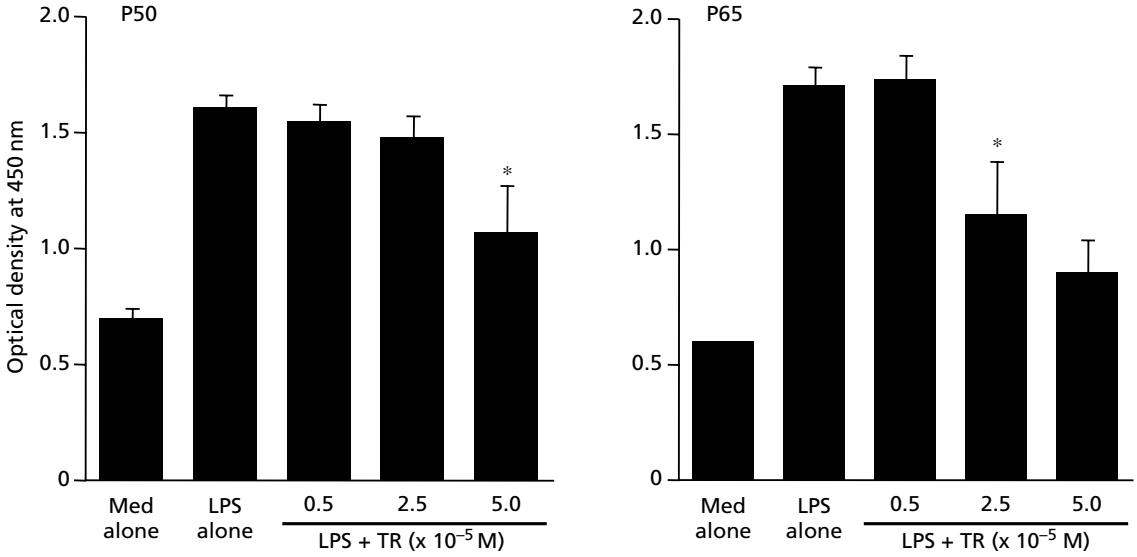

Figure 4 Influence of tranilast (TR) on NF-κB activation in neutrophils by LPS stimulation. Neutrophils prepared from human peripheral blood (1×10^5 cells/mL) were stimulated with 1.0 μg mL^{-1} LPS in the presence of various concentrations of tranilast for 4 h. NF-κB (P50 and P 65) activity in the nucleus was assayed by ELISA. The data are expressed as mean optical density (OD) at 450 nm ± s.e.m. of five different subjects. *$P<0.05$, vs LPS alone.

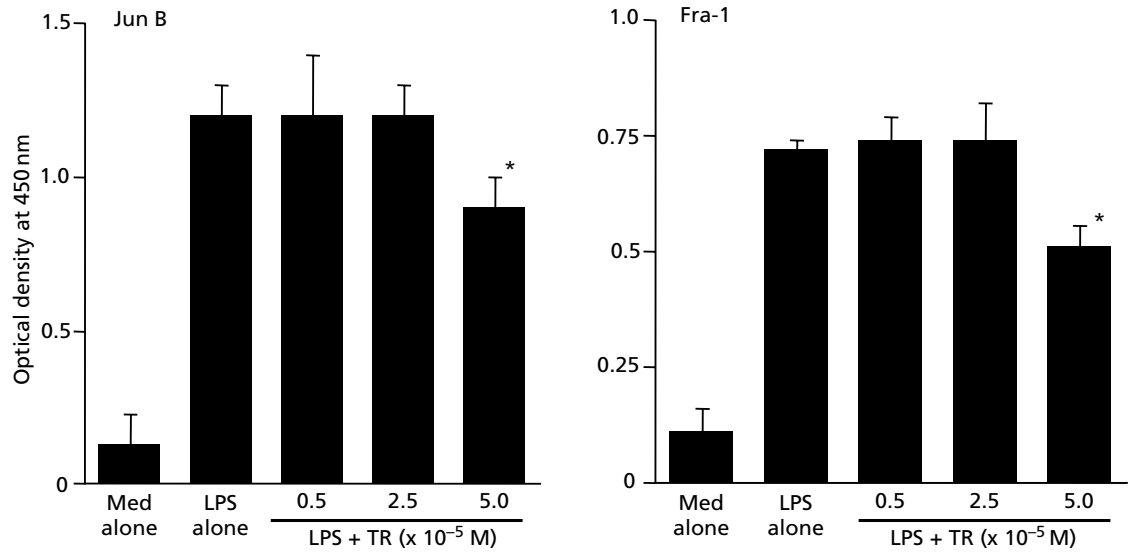

Figure 5 Influence of tranilast (TR) on AP-1 activation in neutrophils by LPS stimulation. Neutrophils prepared from human peripheral blood (1×10^5 cells/mL) were stimulated with 1.0 μg mL^{-1} LPS in the presence of various concentrations of tranilast for 4 h. AP-1 (Fra 1 and Jun B) activity in the nucleus was assayed by ELISA. The data are expressed as mean optical density (OD) at 450 nm ± s.e.m. of five different subjects. *$P<0.05$, vs LPS alone.

The mechanisms and signal transduction pathway involved in the production of MMPs and TIMP in neutrophils are not well defined. Prostaglandins (PGs), especially PGE1 and PGE2, however, have been reported to up-regulate the production of MMPs and TIMP-1 in various human cells after inflammatory stimulation in-vitro (DiBattista et al 1994; Domeij et al 2002). The involvement of PGs is also reported in the production of MMPs in human pulp cells (Huang et al 2004) and prostate epithelial cells (Attiga et al 2000). Our results clearly show the suppressive effect of tranilast on the production of MMPs and TIMP-1, and that the minimum concentration of the agent that causes significant suppression was 5.0×10^{-5} M. These findings suggest that PG-independent mechanism(s) may be implicated in MMP and TIMP-1 production from neutrophils in response to LPS stimulation, since tranilast at 1×10^{-6} M is reported to significantly suppress the production and secretion of PGE2 from human peripheral blood monocytes in response to LPS stimulation in-vitro (Capper et al 2000). The signal transduction pathway involved in MMP production from several types of cells after

inflammatory stimulation was studied using tyrosine kinase and p38 mitogen-activated protein kinase (MAPK) inhibitor, and showed that these kinases are essential factors in the signal pathway for MMP production (Domeij et al 2002; Huang et al 2004). The p38 MAPK pathway also plays an essential role in the production of chemokines such as monocyte chemoattractant protein (MCP)-1 and IL-8 (Matsuyama et al 2004; Lavigne & Eppihimer 2005). Extracellular signal-regulated kinases and Jun N-terminal kinases (JNK) are reported to be implicated in the expression of collagenase and chemokine (IL-8) genes in response to inflammatory stimulation in cultured cells (Chikaraishi et al 2001; Pillinger et at 2003). All of these kinases are recognized to contribute to the activation of transcriptional factors, such as NF-κB and AP-1, which are essential for MMP production (Sato & Seiki 1993; Okamoto et al 1994). From these reports, our results may suggest that tranilast at more than 5.0×10^{-5} M (but not less than 2.5×10^{-5} M) inhibits the activation of NF-κB and AP-1 through the inhibition of several types of kinase activation induced by LPS stimulation and results in suppression of MMP and TIMP-1 production. This suggestion may be supported, in part, by the observation that tranilast could suppress IL-8 release from LPS-stimulated human monocytes by the inhibition of MAPK activation, when the cells were treated with the agent at more than 8.4×10^{-5} M in-vitro (Capper et al 2000). Different from MMPs, which contain binding sites for NF-κB and AP-1, TIMPs have PEA3, a protooncogene-related transcription factor, binding sites in their promoters in addition to AP-1 binding sites and the transcription of TIMPs is dependent on PEA3 and AP-1, which are activated by MAPK (Edward et al 1992). Tranilast at more than 3.0×10^{-5} M is reported to be able to suppress MCP-1 production induced by interleukin-1β stimulation in mesangial cells (Chikaraishi et al 2001), and this inhibitory action of tranilast is owing to its suppressive effect on the activation of JNK, a member of the MAPK family (Chikaraishi et al 2001). These reports may further strengthen our speculation that tranilast could suppress MMP and TIMP-1 production through inhibition of MAPK activation.

In-vitro treatment of neutrophils with tranilast at 5.0×10^{-5} M caused significant, but not complete, suppression of the production of MMPs and TIMP-1 in response to LPS stimulation, whereas the same treatment of cells almost completely inhibited the expression of mRNA for MMPs and TIMP-1. The reason for this discrepancy is not clear at present. It is possible that important events, such as the induction of a certain level of activation of transcription factor and specific mRNA expression, may occur at 0–12 h in neutrophils by LPS stimulation in the presence of 5.0×10^{-5} M tranilast. Then, lower levels of proteins will be secreted and stored in culture medium without degradation for 24 h after stimulation. This may be responsible for the difference between the protein levels measured and mRNA expression. Further experiments are needed to clarify these points.

In conclusion, these results indicate that the therapeutic mode of action of tranilast on neutrophil-derived inflammatory responses may be due to its suppressive action on MMP and TIMP production. Thus, tranilast may reduce the extracellular spread of inflammation through the inhibition of tissue remodelling.

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