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MALFORMIN-A1 INHIBITS THE BINDING OF INTERLEUKIN-1β (IL1β) AND SUPPRESSES THE EXPRESSION OF TISSUE FACTOR IN HUMAN ENDOTHELIAL CELLS AND MONOCYTES

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Abstract—Malformin-A1, a cyclic pentapeptide of microbial origin, antagonized in a competitive manner the binding of ^{125}I-IL1β (interleukin-1β) to human monocytes and cultured human umbilical vein endothelial cells (HUVEC) with IC_{50} values (doses which reduce specific binding by 50%) of 250 ± 80 and 230 ± 25 nM, respectively (N = 3). IL1 increased in a dose-dependent manner the expression of tissue factor, a ubiquitous membrane-anchored glycoprotein that initiates blood coagulation at the surface of HUVEC and human monocytes. Malformin-A1 strongly inhibited IL1-induced tissue factor expression in HUVEC and monocytes with IC_{50} values of 420 ± 35 and 105 ± 25 nM, respectively (N = 3), and reduced IL1-induced expression of intercellular adhesion molecule-1 (ICAM-1, CD54) on HUVEC (IC_{50} = 125 ± 18 nM) (N = 4). These observations demonstrate that malformin-A1 recognizes and blocks IL1β binding to its receptor sites on monocytes and endothelial cells and protects these cells from IL1-induced procoagulant changes.

Key words: malformin-A1; interleukin-1; monocytes; endothelium; tissue factor

TF§ is a ubiquitous membrane-anchored glycoprotein that initiates blood coagulation by forming a complex with circulating factors VII and VIIa [1]. Under normal circumstances, endothelial cells do not express TF but constitutively express thrombomodulin which accelerates thrombin-catalysed activation of protein C, thus contributing to the anticoagulant properties of the endothelium. In some pathological situations, the endothelium or the monocytes, when exposed to inflammatory mediators, can acquire procoagulant properties [2–4]. Indeed, stimulation of these cells by inflammatory compounds such as endotoxin (LPS) or various cytokines alters their antithrombotic properties by inducing the expression of TF and the down-regulation of thrombomodulin, therefore promoting coagulation and thrombosis [2–4]. Among these latter compounds, accumulating evidence suggests that IL1 is a potent inducer of human arterial and venous endothelial cell tissue factor activity [3, 5, 6]. Indeed, recent studies have indicated that IL1 can stimulate endothelial cell tissue factor expression *in vitro* [2, 6] and *in vivo* [5]. IL1 is produced by human monocytes and cultured endothelial cells under certain conditions [7–9], released in a variety of pathological states and thus may be a physiological mediator of endothelial cell perturbation [10]. The pleiotropic effects of IL1 on host physiology suggest that its action must be tightly regulated if it is not to be injurious, suggesting that inhibitors of IL1 action could be useful in the therapy of a variety of inflammatory diseases. Recently, a specific inhibitor of IL1-induced thymocyte proliferation and chondrocyte prostaglandin/collagenase release has been found and cloned [11, 12]. This protein, termed $IL1_{ra}$, has been shown to compete with IL1 for occupancy of IL1 cell surface receptors on various cell types [13–18] and to reduce the severity of sepsis, colitis, arthritis, diabetes and other inflammatory disorders in animals [13–15, 19, 20], suggesting that IL1 may play a primary role in these pathologies.

In the present study, we describe the biochemical properties of malformin-A1, a product of microbial origin [21], that acts as a competitive receptor antagonist with regard to the IL1 receptor on human monocytes and HUVEC. We further determined its activity with regard to IL1-induced tissue factor expression on these cells.

MATERIALS AND METHODS

Materials. Heparin (sodium salt from porcine intestinal mucosa), ECGS, malformin-A1, *o*-phenylenediamine dihydrochloride and BSA (Fraction V) were purchased from Sigma Chemical Co. (France). Human recombinant tissue thromboplastin (Dade) was from Baxter Diagnostics Inc. (U.S.A.). PPSB was from Intertransfusion (France). Substrate S2222 was from Kabi (Sweden). ^{125}I-IL1β (1600 Ci/

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§ Abbreviations: ECGS, endothelial cell growth supplement; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL1, interleukin-1β; $IL1_{ra}$, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; TF, tissue factor.

mmol) was from NEN-Dupont (France). IL1β and IL1α were provided by British Biotechnology Ltd (U.K.). Fetal calf serum and all tissue culture reagents were from Boehringer Mannheim (Germany).

^{125}I-IL1 binding to human monocytes and HUVEC. Human mononuclear cells were prepared according to Boyum [22] from freshly drawn venous blood in a 1 USP/mL heparin solution. Blood was diluted in 1 vol. of PBS without calcium or magnesium and centrifuged (400 g, 30 min, 20°) on Ficoll-paque (Pharmacia). The supernatant fluid was discarded and the pelleted monuclear cells were finally suspended (1×10^8 cells/mL) in PBS. The cell population consisted of more than 95% mononuclear cells as judged by morphological observation. Experiments on the specific binding of ^{125}I-IL1β to cells and its inhibition by receptor antagonists were performed as previously described [18]. Incubations were carried out in a total 0.5 mL vol. of binding buffer (RPMI 1640 supplemented with 1% BSA, 20 mM HEPES, pH 7.2) which contained ^{125}I-IL1β (0–2.0 nM for saturation experiments or 0.2 nM for competition studies). Triplicate incubations were carried out for 120 min at 4° and terminated by the addition of a 3 mL ice-cold assay buffer. Cells were then centrifuged (600 g, 10 min) and washed twice with 2 mL ice-cold incubation buffer and radioactivity was measured by scintillation counting. Non-specific binding was defined as the total binding measured in the presence of excess unlabelled IL1β (0.5 μM) and specific binding as the difference between total binding and non-specific binding. Binding of ^{125}I-IL1β to confluent HUVEC (5×10^5 cells/well) was performed in a similar manner. The percent inhibition was expressed as: %I = (total binding − total binding with antagonist)/specific binding × 100. The IC_{50} value was defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding and determined with an indirect Hill plot [23]. The equilibrium inhibition constant (K_i) was calculated from the Cheng and Prusoff [24] equation: $K_i = IC_{50}/(1 + L/K_D)$, where L is the concentration of labelled ligand and K_D the apparent dissociation constant of radioligand binding to monocytes. The apparent dissociation constants (K_D) and the maximal number of binding sites (B_{max}) were calculated using a Scatchard representation of the experimental data [25]. Data from saturation and competition studies were analysed using a non-linear regression program [26].

Determination of tissue factor activity on the cells. HUVEC (passage 3–10) were isolated and cultured as described [27] in 96-well microplates in RPMI 1640 medium supplemented with 10% fetal calf serum, ECGS (30 μg/mL) and heparin (100 μg/mL). Mononuclear cells were obtained from human heparinized blood as described above. Procoagulant activity was assayed according to Suprenant *et al.* [28]. Briefly, adherent cells (2×10^5 HUVEC/well and 5×10^3 monocytes/well) were incubated for 6 hr at 37° in M-199 with IL1β in the absence or presence of the indicated concentrations of malformin-A1. The medium was removed and wells were washed twice with 1 mL of PBS and incubated for 45 min at 37° with 250 μL of M-199 containing PPSB (0.44 U/mL FVII) and 100 μg/mL of substrate S2222. O.D. was measured at 405 nm. TF activity was obtained from a standard curve (log [ΔO.D.$_{405}$/min] vs log [U/mL]) using serial dilutions of human recombinant tissue thromboplastin in M-199 assayed as described above. Undiluted thromboplastin was arbitrarily assigned a value of 1 U/mL. TF activity was normalized to the cell counts from the same well and expressed as μU of TF/10^5 cells.

Induction and detection of cell surface ICAM-1. The expression of ICAM-1 was determined by ELISA. HUVEC ($2–5 \times 10^5$ cells/well) were seeded in 96-well tissue culture plates 48 hr before the experiment. The experiments were performed in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics. After incubation for 24 hr at 37° with IL1 (10 ng/mL) in the presence of malformin-A1, ICAM-1 expression was determined using mAb followed by goat anti-mouse peroxidase (British Biotechnology Ltd, Oxford, U.K.) and *o*-phenylenediamine dihydrochloride as substrate. The plates were read on a microtitre plate reader at 492 nm. All data were given as the mean ICAM-1 concentration ± SD from quadruplicate wells calculated on the basis of a standard curve determined in parallel.

RESULTS

Effect of malformin-A1 on the binding of ^{125}I-IL1β to monocytes and HUVEC

As already shown [17, 18], total binding of ^{125}I-IL1β to monocytes and HUVEC at 4° was reversible, time dependent and saturable (Fig. 1). For monocytes, a Scatchard analysis of the bound/free ratio of the radiolabelled ^{125}I-IL1 vs bound ^{125}I-IL1 revealed the presence of one class of non-interacting binding sites exhibiting high affinity with an apparent equilibrium dissociation constant (K_D) value of 0.68 ± 0.08 nM and a total number of receptor sites (B_{max}) of 43 ± 12 fmol/10^7 cells (3800 ± 1100 sites/cell) (N = 3). Under the same experimental conditions, ^{125}I-IL1β bound to HUVEC with a K_D value of 0.2 ± 0.05 nM and a maximum binding capacity of 21.0 ± 9.4 fmol/10^7 cells (N = 6) (Fig. 1B). As shown in Fig. 2, unlabelled IL1β and IL1α displaced ^{125}I-IL1β specifically bound to its high affinity receptor sites on monocytes in a dose-dependent manner. The concentrations required to inhibit 50% of the specific binding (IC_{50}) were 0.5 ± 0.07 and 120 ± 37 nM, respectively. With regard to HUVEC, IL1β and IL1α displaced ^{125}I-IL1β specifically bound to its high affinity receptor sites with IC_{50} values of 1.2 ± 0.9 and 31 ± 9.4 nM, respectively. Despite minor differences in experimental conditions, our results confirm those already published concerning the ability of these compounds to displace ^{125}I-IL1β from its high affinity binding sites on these cells [17, 18]. Under the same experimental conditions, malformin-A1 dose-dependently inhibited the specific binding of ^{125}I-IL1β to monocytes and HUVEC with IC_{50} values of 250 ± 80 and 230 ± 25 nM, respectively (N = 3). Using the Cheng and Prusoff relationship [24], the inhibition constants (K_{Di}) for malformin-A1 were 193 ± 62 and 332 ± 53 nM for monocytes and HUVEC, respectively. It is noteworthy that whether mal-

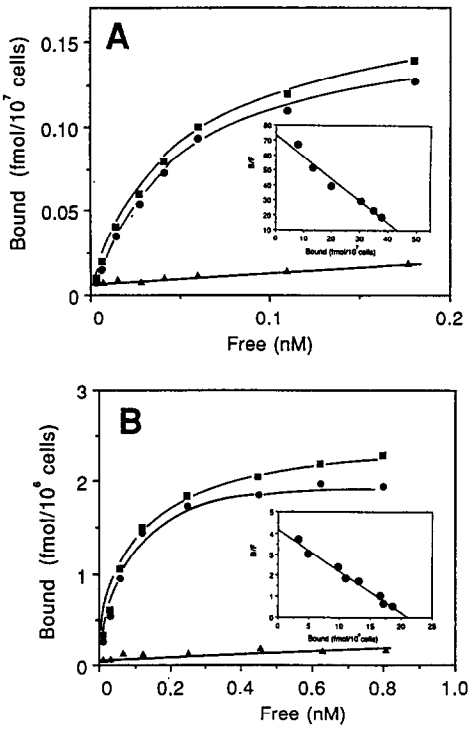

Fig. 1. Specific binding of ^{125}I-IL1β to human monocytes and HUVEC. Monocytes (2×10^7 cells/mL) (**A**) or confluent HUVEC (5×10^5 cells/well) (**B**) were incubated for 120 min at 4° with increasing concentrations of ^{125}I-IL1β. Specific binding (●) is given by the difference between total (■) and non-specific binding (▲) determined in the presence of 0.5 μM of ^{125}I-IL1β. Insets: Scatchard plots of the specific binding of ^{125}I-IL1β calculated from saturation isotherms. Each point is the average of results from at least three independent experiments performed in triplicate.

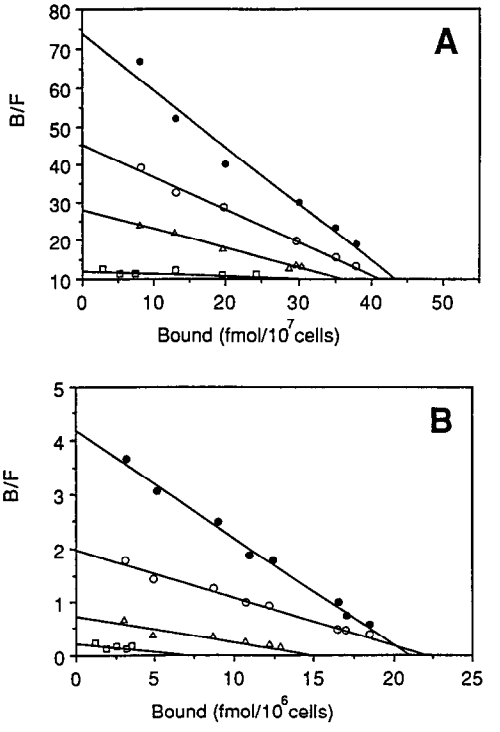

Fig. 3. Nature of the inhibitory effect of malformin-A1 on the binding of ^{125}I-IL1β to monocytes and HUVEC. Monocytes (2×10^7 cells/mL) (**A**) or confluent HUVEC (5×10^5 cells/well) (**B**) were incubated with increasing concentrations of ^{125}I-IL1β in the absence (●) or presence of malformin-A1 [125 nM (○); 250 nM (△) or 500 nM (□)]. Scatchard representations of the experimental data were determined from saturation curves as described under Materials and Methods. Results are expressed as the mean of triplicate determinations.

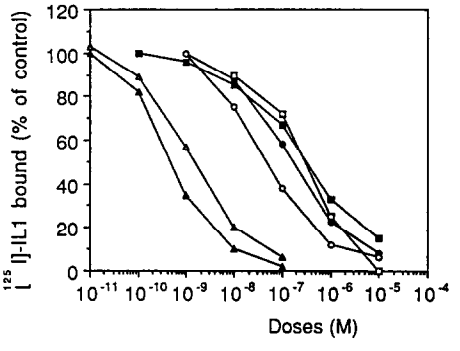

Fig. 2. Effect of malformin-A1 on the binding of ^{125}I-IL1β. Human monocytes (2×10^7 cells/mL) (full symbols) or HUVEC (5×10^5 cells/well) (empty symbols) were incubated with 0.2 nM of ^{125}I-IL1β for 120 min at 4° in the presence of increasing concentrations of IL-1β (▲, △), IL1α (●, ○) or malformin-A1 (■, □). Each data point is the average of results from at least three independent determinations performed in triplicate.

formin-A1 was pre-incubated with the cells or the radiolabelled ligand, a similar inhibitory effect was obtained (not shown). In order to determine the nature of the antagonism of malformin-A1 on ^{125}I-IL1β binding to monocytes or HUVEC, cells were incubated for 120 min at 4° with increasing concentrations of ^{125}I-IL1β (0–2 nM) in the absence and presence of malformin-A1 (125, 250 and 500 nM) (Fig. 3). The Scatchard analysis indicated that this compound was a competitive IL1β receptor antagonist. Indeed, for both cell types, the maximal number of receptor sites (B_{max}) was not affected, whereas the apparent dissociation constant (K_D) values increased with respect to the increasing concentrations of malformin-A1 (Fig. 3). In a Shild analysis, log (K'_D/K_D-1) was plotted, where K'_D and K_D are apparent and equilibrium dissociation constants of ^{125}I-IL1β in the presence and absence of antagonist, respectively, vs log (antagonist). A unity slope was found, therefore showing that the blockade of ^{125}I-IL1β binding by malformin-A1 on monocytes and HUVEC was consistent with a simple competitive antagonism.

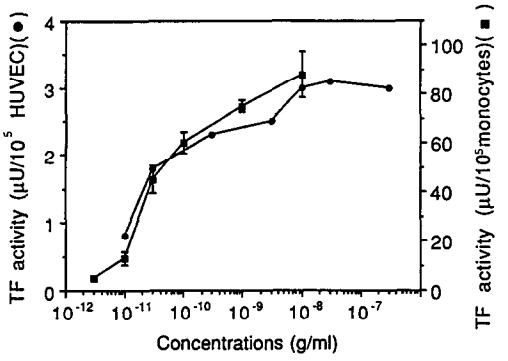

Fig. 4. Effect of IL1β on TF expression in HUVEC and monocytes. Human monocytes (■) and HUVEC (●) were incubated for 6 hr with the indicated concentrations of IL1β. Tissue factor expression was determined as described under Materials and Methods. Results are expressed as a mean ± SD (N = 6).

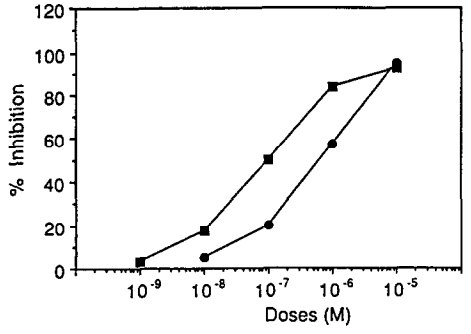

Fig. 5. Effect of malformin-A1 on IL1β-induced TF expression in HUVEC and monocytes. Increasing concentrations of malformin-A1 were incubated with HUVEC (●) or human monocytes (■) in the presence of IL1β (10 ng/mL). Results are expressed as % inhibition of the control response (N = 6).

Effect of malformin-A1 on IL1β-induced TF expression on cells

Exposure of confluent HUVEC or adherent monocytes to increasing concentrations of IL1β resulted in a significant, dose-dependent increase in TF expression on the cell surface (Fig. 4). After incubation with the highest doses of IL1β, human monocytes exhibited higher maximal levels of TF expression compared to pyrogen-stimulated HUVEC. Indeed, a ≈30-fold difference in TF expression level was observed between endothelial cells and monocytes.

As shown in Fig. 5, malformin-A1 counteracted in a dose-dependent manner IL1β-induced TF expression in HUVEC and monocytes. The IC_{50} values (concentrations which inhibited 50% of the IL1β-induced TF expression) were 420 ± 35 nM and 105 ± 23 nM for HUVEC and monocytes, respectively.

Effect of malformin-A1 on IL1β-induced ICAM-1 expression in HUVEC

As shown in Table 1, IL1 induced the expression of a high level of ICAM-1 (CD54) on the surface of HUVEC, whereas almost no ICAM-1 was detected in unstimulated HUVEC. Malformin-A1, up to 10 μM, had no effect on ICAM-1 expression in resting cells but strongly decreased the expression of ICAM-1 in IL1-stimulated endothelial cells at a dose of 1 μM (Table 1). Malformin-A1 inhibited IL1-induced ICAM-1 expression in a dose-dependent manner with an IC_{50} value of 125 ± 18 nM.

DISCUSSION

Vascular endothelial cells and monocytes play an active role in the regulation of blood coagulation [29]. The haemostatic balance of the surface of these cells is affected by bacterial endotoxins and the immunomodulators IL1 and tumour necrosis factor-α which have been shown to down-regulate thrombomodulin surface activity [5, 30, 31] and induce TF expression [2–4]. These effects may contribute to the prothrombotic vascular changes which are associated with inflammatory reactions or which occur in various pathological states such as cancer or sepsis where the haemostatic balance of endothelial cells and monocytes is strongly affected.

Recent studies have shown that IL1, a potent mediator of immunologic and inflammatory phenomena [32] released in a variety of pathological states, may be an important physiologic mediator of endothelial cell perturbation. These considerations have been further emphasized by other studies indicating that IL1 can stimulate endothelial cells *in vitro* [2, 3, 6] and *in vivo* [5], causing them to acquire procoagulant and proadhesive properties. These studies indicate that IL1 can shift the balance of procoagulant and anticoagulant reaction of the endothelium unidirectionally, favouring clot formation. The surface of the perturbed endothelium could thus provide a template, facilitating the development of a prethrombotic state, and provide a model system for the early stages of thrombosis.

In this report, we have confirmed that IL1 acts on human vascular endothelium and mononuclear cells to induce the expression of tissue factor. This effect of IL1, like that previously reported [2, 3, 6], was found to be concentration dependent, time dependent, reversible and blocked by inhibitors of protein or RNA synthesis (not shown). Although pyrogen-induced prohaemostatic changes on the endothelial cell surface have been reported for several years, a substance that effectively counteracts these effects has so far not yet been described. In the present study, malformin-A1 was investigated for such activities. This compound, a cyclic pentapeptide of microbial origin [21], was demonstrated to interfere with the binding of ^{125}I-IL1 to human monocytes and HUVEC *in vitro*. Indeed, malformin-A1 inhibited ^{125}I-IL1 binding to human monocytes and HUVEC with IC_{50} values of 250 ± 80 and 230 ± 25 nM, respectively. Malformin-A1 was therefore 10-fold less active than the only IL1 receptor antagonist described to date ($IL1_{ra}$)

Table 1. Effect of malformin-A1 on IL1β-induced ICAM-1 expression in HUVEC

Compounds	Dose (ng/mL)	ICAM-1 concentration (ng/10^5cells)
Unstimulated		0.5 ± 0.1
Controls		6.1 ± 0.9
Malformin-A1	1000	0.6 ± 0.3
	300	1.8 ± 0.2
	100	2.7 ± 0.5
	30	6.0 ± 0.4

Confluent HUVEC (2–3 × 10^5 cells/well) were incubated for 24 hr with IL1β (10 ng/mL) in the presence of malformin-A1 at the indicated concentrations. ICAM-1 expression was determined as described under Materials and Methods and expressed as a mean ± SD of quadruplicate wells.

[11, 12, 16]. Early experiments with recombinant human $IL1_{ra}$ suggested that it could not bind to and therefore could not block IL1 action on cells expressing the type II receptor. Since this receptor has been found on a wide variety of cell types including B-cells, polymorphonuclear leucocytes and monocytes, these results imply that $IL1_{ra}$ might not regulate IL1 action on these cells. The results reported here and elsewhere [17] have disproved the fundamental premise that $IL1_{ra}$ cannot bind to the type II IL1 receptor on these cells. However, although our binding data directly addressed this question, we note that $IL1_{ra}$ did not inhibit the effect of IL1 on TF expression in monocytes, whereas it strongly reduced IL1-induced procoagulant changes in HUVEC ($IC_{50} = 14 \pm 1.9$ nM). In contrast, malformin-A1 was found to counteract IL1-induced TF expression in both HUVEC and monocytes. These results, combined with our binding data, suggest that malformin-A1's receptor antagonist properties apply to the type II as well as the type I IL1 receptor but remain unsatisfactory, since neither compound exhibited the same pattern of inhibition with regard to IL1-induced TF expression in HUVEC and monocytes. Such an observation has already been made by several authors using other experimental models; they suggest that $IL1_{ra}$ might interfere differently with both type I and II IL1 receptors depending on the maturation, differentiation and activation state of the cells [17].

The appearance of monocytes at inflammation sites is the result of a coordinated series of events. These steps may include the release of cells from bone marrow into the circulation, their adherence to endothelial cells and their subsequent migration into adjacent tissues. Among these events, adhesion of monocytes to endothelial cells is a crucial step in cell accumulation [33, 34]. Adhesion is augmented by IL1 through increased expression of adhesion glycoproteins on endothelial cells [34, 35]; and $IL1_{ra}$ pretreatment of endothelial cells blocks this response [35]. Our results also demonstrate that malformin-A1 blocks the expression of ICAM-1 (CD54), a glycoprotein shown to play a major role in the adhesion of monocytes, lymphocytes and neutrophils to the endothelium [36]. The ability of malformin-A1 to inhibit IL1-induced ICAM-1 expression suggests that in this model as well malformin-A1 acts as a potent inhibitor of the biological effects of IL1 but does not preclude the importance of IL1 as a participant in this response, shown to implicate a wide variety of other mediators [37]. Therefore, definite evidence for IL1 importance in these phenomena will be determined by the effect of malformin-A1 in a more "global" model of inflammation.

Since IL1 has been shown to assist in B-cell activation [38] and proliferation [39], malformin-A1 is potential therapy for B-cell leukaemias [40] and lymphomas, multiple myeloma [41], insulin-dependent diabetes, systemic lupus erythematosus [42] and some forms of glomerulonephritides. Since IL1 can act synergistically with other cytokines in inducing stem cell proliferation [43], granulocytic leukaemias [44, 45], rheumatoid arthritis [46] and gout might also respond to malformin-A1. Furthermore, pyrogen-induced prohaemostatic changes at the vessel wall are thought to play a role in the pathogenesis of thrombotic microvessel obstruction. It is also known that malignancy and acute phase reactions [47], situations where systemic effects of cytokines such as IL1 or tumour necrosis factor-α are found, are frequently associated with thromboembolic diseases. Changes in the haemostatic activities of the vascular wall, favouring prothrombotic mechanisms, may be of pathogenetic importance for macrovessel thrombosis in such settings. Therefore, since malformin-A1 seems to be capable of completely neutralizing the pyrogen-mediated decrease in thromboresistance of the vascular wall, it is likely that it may also be of biological significance in limiting the procoagulant effect of inflammatory immunomodulators in the vascular wall.

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