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INHIBITION OF PRODUCTION OF MACROPHAGE-DERIVED ANGIOGENIC ACTIVITY BY THE ANTI-RHEUMATIC AGENTS GOLD SODIUM THIOMALATE AND AURANOFIN

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SUMMARY We have investigated the effect of gold sodium thiomalate and auranofin, gold compounds employed in the treatment of rheumatoid arthritis, on production of macrophage-derived angiogenic activity. Elicited mouse peritoneal macrophages were cultured in the presence or absence of gold compounds or thiomalic acid, and the macrophages or their conditioned media were then assayed for their angiogenic activity in rat corneas. Control macrophage conditioned medium was potently angiogenic. In contrast, conditioned medium from gold or thiomalic acid treated macrophages was not. Addition of gold compounds or thiomalic acid to control macrophage conditioned medium did not inhibit its angiogenic activity. Drug treatments did not significantly affect macrophage lactate dehydrogenase release, lysozyme release, or protein synthesis. We conclude that gold sodium thiomalate and auranofin potently reduce the detectable angiogenic activity produced by macrophages. © 1988 Academic Press, Inc.

Angiogenesis, the growth and proliferation of new blood vessels, is a prominent feature of fibroproliferative processes such as tumor growth, wound repair, and inflammation (1-5), and is an integral part of the growth of the inflammatory, invasive rheumatoid synovial pannus (6). Monocytes and macrophages play a critical role in mediating these angiogenic processes (7). Polverini et al. first showed that macrophages obtained from the peritoneal cavity of mice and guinea pigs were potently angiogenic (4). We have previously shown that a subpopulation of macrophages isolated directly from the human rheumatoid synovial pannus induced an angiogenic response in the in vivo rat corneal model (7).

Inhibitors of the angiogenic process may prove useful in the treatment of fibroproliferative disorders such as rheumatoid arthritis. Gold compounds, such as gold

Abbreviations

DMEM, Dulbecco's modified Eagle's medium; MCM, Macrophage conditioned medium; LPS, Lipopolysaccharide; MDAA, Macrophage-derived angiogenic activity; GST, Gold sodium thiomalate.

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sodium thiomalate (GST) and auranofin are frequently used in the treatment of rheumatoid arthritis, but their mechanism of action is unclear. These compounds have been shown to have several inhibitory effects on macrophage function, including inhibition of antigen presentation, collagenase production, and complement C2 production (8-12). We hypothesized that gold compounds may mediate their effects by modulating macrophage-mediated angiogenesis. In this study, we have investigated the effect of these compounds on the production of macrophage-derived angiogenic activity (MDAA) using the in vivo rat corneal bioassay. Our results show that both GST and auranofin potently reduce or completely inhibit the angiogenic response without altering macrophage viability, constitutive lysozyme release, or generalized protein synthesis. These studies may provide a new explanation for the mechanism of action of gold compounds.

METHODS

Preparation of Macrophages and Macrophage conditioned medium (MCM)

Eight week old Balb/c mice were injected with mineral oil (Drakeol 6 VR, Penreco, Butler, PA) or Brewer's thioglycollate (Difco, Baltimore, MD), and their elicited peritoneal macrophages harvested four to five days later as previously described (13). These macrophages are potently angiogenic in the rat corneal bioassay (4). Macrophages were plated at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum plus gentamicin and allowed to adhere to 75 mm culture flasks (Costar, Cambridge,MA) for one hour. After adherence, the culture medium was replaced by an equal volume of medium (10 ml) containing 0.5% fetal calf serum with and without various concentrations of GST, thiomalic acid, or auranofin. In the case of auranofin, which is toxic to cells if left in culture for prolonged periods, the drug-containing medium was washed off after one hour and replaced with fresh medium containing 0.5% fetal calf serum without auranofin for an additional 48 hour incubation. The MCM was concentrated ten fold using Centricon 10 filters (Amicon, Danvers, MA). Since the production of MDAA is enhanced by lipopolysaccharide (LPS), in some cases LPS (phenol- extracted preparation of E. Coli 055:B5, Sigma, St. Louis, MO.) ($5\mu g/ml/10^6$) cells was added to the cultures for a twenty hour incubation, the cells washed extensively, and then incubated with gold compounds as described above in the absence of LPS.

Rat corneal bioassay for angiogenesis

MCM concentrated ten fold (5.0 μl) was incorporated into an equal volume of slow-release Hydron (Interferon Sciences, New Brunswick, NJ) and 10-μl pellets were implanted asceptically into a pocket within the rat corneal stroma (14). In some cases, macrophages (5×10^5) preincubated with GST were implanted directly in the rat corneas. Corneas were examined daily for seven days with a stereomicroscope and perfused with colloidal carbon at the end of the observation period to provide a permanent record of the angiogenic response (15).

Viability assessment

Viability of the macrophages exposed to the gold compounds was assessed by cellular trypan blue exclusion and by lactate dehydrogenase release into the MCM. Lactate dehydrogenase was measured using a commercially available procedure (Sigma, St. Louis, Mo.).

Lysozyme measurement

Constitutive lysozyme release was measured using unconcentrated MCM by lysis of micrococcus lysodeikticus suspended in agarose using a commercially available procedure

(Kallestad, Chaska, MN). Hen egg white lysozyme was used as a reference standard (Sigma,St. Louis, MO.) (16).

Measurement of the effect of gold compounds on macrophage protein synthesis

Assessment of protein synthesis was done using a modification of previously described methods (9). After incubation with the gold compounds, duplicate cultures of macrophages were incubated with leucine-free DMEM for one hour at 37°C. Fifty uCi/ml [^{3}H] leucine (Amersham, Arlington Heights, IL) were added to $5x10^6$ cells for a further one hour incubation. Macrophages ($5x10^6$/2 ml DMEM) were subsequently lysed with lml 1M sodium hydroxide, and the cell lysate added to 2ml 5% trichloracetic acid. After heating at 75°C for thirty minutes, precipitation was allowed to proceed overnight at 4°C. The precipitates were pipetted in triplicate onto glass fiber filters, washed with 95% ethanol and counted in a scintillation counter (Beckman, Irvine, CA).

RESULTS

Table 1 shows the cumulative results of the effect of incubation of mouse peritoneal macrophages with gold compounds. Conditioned media from unstimulated or LPS-stimulated mouse peritoneal macrophages were potently angiogenic. Figure 1 shows a

Table 1

EFFECTS OF ANTI-RHEUMATIC DRUGS ON PRODUCTION OF MDAA

Test group	Number of corneas assayed	
Conditioned medium from:	Positive Angiogenic responses	Negative Angiogenic responses
Control macrophages (mϕ's)	3	0
Control mϕ's incubated with LPS*	6	0
Mϕ's treated with GST (2 μg/ml)	0	4
Mϕ's treated with GST (33 μg/ml)	1	5
Mϕ's treated with thio-malic acid (0.76 μg/ml)	0	3
Mϕ's treated with thio-malic acid (12.5 μg/ml)	0	3
LPS stimulated mϕ's treated with thiomalic acid (0.76 μg/ml)	0	3
LPS stimulated mϕ's treated with thio-malic acid (12.5 μg/ml)	0	3
Mϕ's treated with aurano-fin (0.1 μg/ml)	1	3
LPS stimulated mϕ's with GST (2μg/ml) added	3	0
LPS stimulated mϕ's with thiomalic acid (0.76 μg /ml) added	3	0
LPS stimulated mϕ's with auranofin (0.1 μg /ml) added	3	0
Mϕ's pre-incubated with GST (50 μg/ml); Mϕ's injected into corneas	7	0

* Mϕ's were incubated with 5 μg/ml LPS for twenty hours. After incubation, the LPS was vigorously washed off and fresh tissue culture medium containing test drugs was added.

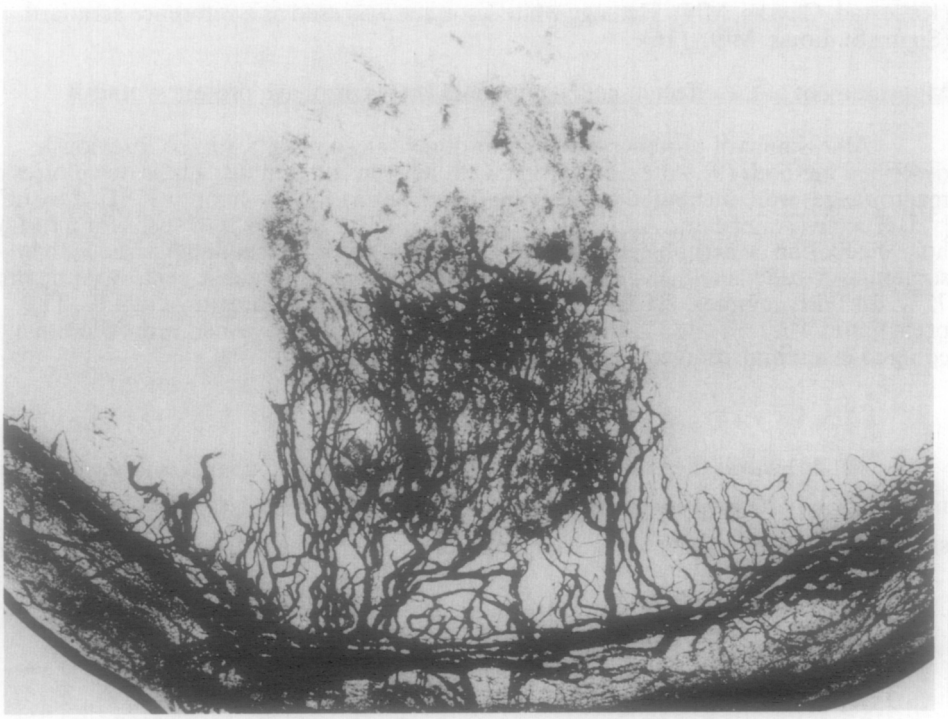

Figure 1 shows a positive angiogenic response induced by control MCM. New blood vessel growth from the corneal limbus toward the Hydron implant at the top of the picture can be seen.

positive angiogenic response induced by MCM. Figure 2 shows a negative corneal response from MCM obtained from GST treated macrophages. Treatment of macrophages with 2 μg/ml or 33μg/ml GST resulted in inhibition of the production of MDAA. Incubation of macrophages with equivalent doses of thiomalic acid (0.76 and 12.5 μg/ml) also resulted in inhibition of production of MDAA. If the macrophages were preincubated with LPS, which

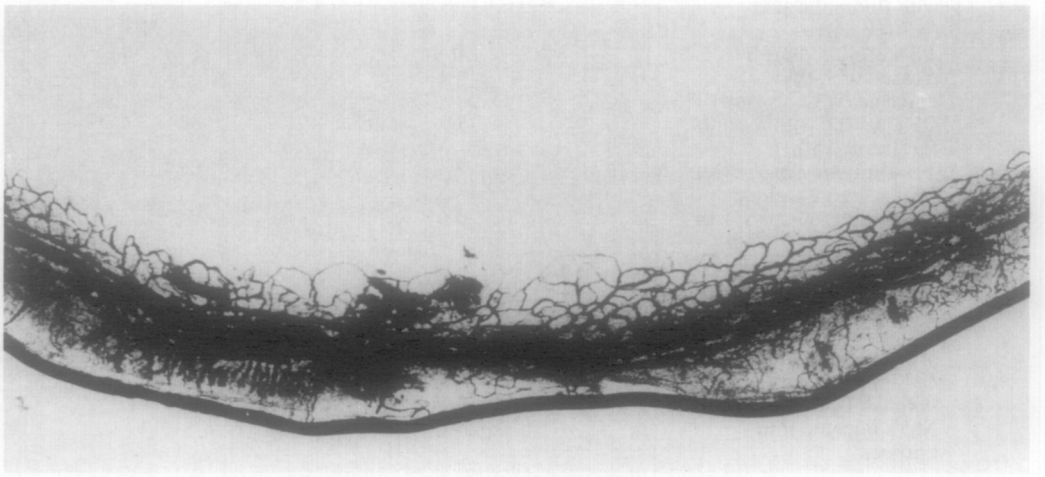

Figure 2 shows a negative angiogenic response when a Hydron implant containing MCM from GST treated (2 μg/ml) mϕ's was implanted in the rat cornea.

stimulates the production of MDAA, the normally angiogenic response was lessened by incubation with thiomalic acid, but not completely abolished. In order to determine whether the continuous presence of GST on macrophages was necessary to inhibit production of MDAA, macrophages were preincubated with GST (50 μg/ml) for 48 hours, washed extensively, and implanted into rat corneas. These macrophages implanted in the cornea and free of the presence of GST induced an angiogenic response, indicating that they regained their angiogenic ability.

Treatment of macrophages with auranofin (0.1 μg/ml) also inhibited the production of MDAA. In this case, macrophages were preincubated with auranofin for 1 hour., and then incubated in the absence of drug for the preparation of conditioned medium. As has been observed previously, continuous incubation with auranofin results in significant cytotoxic effects (8). Thus, while the continuous presence of GST and thiomalic acid was required to inhibit production of MDAA, a one hour pretreatment of macrophages with auranofin was sufficient to inhibit MDAA production.

To ensure that the gold compounds and thiomalic acid were acting directly on the macrophages, rather than (a) inhibiting or inactivating MDAA in the MCM, or (b) acting on other components of the angiogenic response, such as endothelial cells, 2 μg/ml GST, 0.76 μg/ml thiomalic acid or 0.1 μg/ml auranofin were added to control MCM prior to corneal implantation. Under these conditions, no inhibition of the angiogenic response was seen. In order to determine whether drug treatments impaired the viability of the macrophages, viability was assayed by measurement of trypan blue exclusion and lactate dehydrogenase release from cultured cells. Greater than ninety percent of the cells excluded dye in all cases. Similarly, lactate dehydrogenase release was not altered between control and drug treated macrophages. The amount of lactate dehydrogenase released by untreated and drug treated macrophages was less than 10% of that found by lysis of control macrophages. Release of lysozyme, a constitutive product of macrophages, was not markedly altered by drug treatment.

General protein synthesis by macrophages, as measured by uptake of [^{3}H] leucine is shown in fig.3. Protein synthesis was not appreciably altered by treatment with 2 μg/ml GST or 0.1 μg/ml auranofin. GST (33 μg/ml) reduced [^{3}H] leucine incorporation, by less than 25%, as did thiomalic acid (0.76 μg/ml and 12.5 μg/ml).

DISCUSSION

The concentrations of GST obtained therapeutically in vivo are generally accepted to be in the range of 4-10 μg/ml in serum, with the level in synovial tissue reaching about 42-50 μg/ml, due to sequestration in synovial cells and macrophages (25). Concentrations of auranofin in blood are generally in the range of 0.3-1.0 μg/ml, with higher levels in synovial tissue. In this study we have shown that GST and auranofin, at doses lower than or equivalent to those attained therapeutically in humans in vivo, potently inhibited the production of MDAA. The concentrations of both GST and auranofin required to inhibit

production of MDAA are lower than those necessary to inhibit production of other macrophage products, such as complement C2 or collagenase. This effect, in the case of GST, appears to be at least in part due to the thiomalic acid moiety. However, whether this is a specific effect of thiomalic acid, or rather, due to non-specific effects of free thiol groups, is not yet clear. In our experiments, direct inhibition of angiogenesis in vivo was not observed with GST and auranofin. Rather these drugs acted on the macrophages in culture to inhibit their production of angiogenic activity. In the corneal bioassay system, adding drugs back to potently angiogenic MCM did not inhibit the angiogenic response. The continual presence of GST is necessary for this inhibition of macrophage production of angiogenic activity, since macrophages preincubated with GST were potently angiogenic when implanted in corneas, despite their prior drug treatment. With auranofin, on the other hand, a one hour preincubation was sufficient to inhibit the subsequent production of angiogenic activity by treated macrophages. These drugs appear to exert their action on macrophages even at doses that do not markedly affect their viability, general protein synthesis, or lysozyme secretion.

The mechanism of the inhibition of production of MDAA in response to the drugs used in this study is unclear. It seems likely that gold compounds inhibit the secretion of angiogenic substance(s). Gold compounds have been shown to inhibit monocyte production of a variety of factors such as complement C2, and interleukin-1 (11,12,26). One of the main

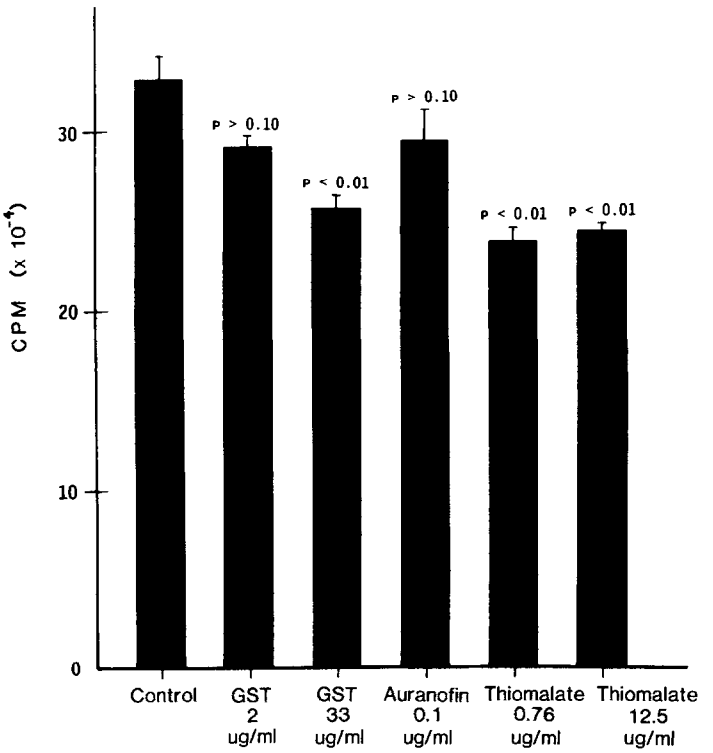

Figure 3 shows the uptake of [^{3}H] leucine by mϕ's (see methods section). Results represent the mean ± s.e. for 6 determinations. Significance values were determined using Student's t-test, comparing experimental to control.

angiogenic factors liberated by macrophages has been shown by Leibovich, et al to be tumor necrosis factor-alpha (27). Studies are currently in progress to evaluate whether gold compounds inhibit the production of specific inducible proteins such as tumor necrosis factor-alpha. It is also possible, however, that macrophages incubated with these drugs do not produce detectable angiogenic activity due to the increased production of an inhibitor of angiogenesis.

Several inhibitors of the angiogenic process have been described to date. These include factors from adult cartilage (18, 19) and bovine vitreous (20), both of which contain potent protease inhibitors. A placental ribonuclease inhibitor has been observed that abolishes both the angiogenic and ribonucleolytic activities of the putative angiogenic protein, angiogenin (20). Protamine, a basic protein from fish sperm, inhibits angiogenesis, possibly by binding heparin and blocking the linear migration of capillary endothelial cells (22). Angiostatic steroids such as 11-α-epihydrocortisol, which have little or no glucorticoid or mineralocorticoid function, have been found to inhibit angiogenesis in the presence of heparin (23,24). The antineoplastic agents, mitoxantrone and bisantrene, have been shown to inhibit angiogenesis in the rat cornea and may act by inhibiting prostaglandin biosynthesis (25). Direct inhibition of endothelial cell proliferation in culture by GST at concentrations as low as 1 μg/ml, and by 0.1 μg/ml auranofin has been reported (17). This study, unlike ours, examined endothelial cell proliferation in vitro, rather than the process of angiogenesis in vivo.

Drugs that inhibit the production of angiogenic substances may prove useful in the therapy of disease states, such as rheumatoid arthritis, in which angiogenesis plays a prominent role. To our knowledge, GST and auranofin are among the first compounds which have been shown to act directly on the macrophage to cause a decrease in the production of angiogenic activity.

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