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### Research Article

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# Inhibition of Human Endothelial Cell Proliferation by Gold Compounds

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## Abstract

Neovascularization has a role in the propagation of rheumatoid synovitis because the spread of mononuclear cell infiltration and the growth of pannus are dependent on the growth of new blood vessels. Growth of such vessels requires local endothelial cell (EC) proliferation. Inhibition of synovial EC proliferation, therefore, would have the potential to diminish rheumatoid inflammation. We have, therefore, studied the effects of gold sodium thiomalate (GST), auranofin, and gold chloride on the proliferation of human umbilical vein EC. GST suppressed both basal and EC growth factor-induced tritiated thymidine incorporation into EC in a dose-dependent fashion. Inhibition was observed with concentrations as low as 1  $\mu\text{g/ml}$  GST, 5  $\mu\text{g/ml}$  gold chloride, and 0.1  $\mu\text{g/ml}$  auranofin, levels attainable in blood and synovium of patients. These results suggest that gold compounds have an antiangiogenic effect. The low concentrations inhibiting EC proliferation suggest that gold compounds may suppress rheumatoid synovitis by reducing the number of small blood vessels available for mononuclear cell infiltration and synovial tissue proliferation.

## Introduction

Angiogenesis is the process of generation of new blood vessels. It is required not only in normal phenomena such as embryonic development and wound repair but also in pathologic states such as tumor growth, connective tissue proliferation, chronic inflammation, and certain immune reactions (1). In the rheumatoid synovial membrane, mononuclear cells in the sublining region of the synovium carry out a series of cellular and humoral immune reactions responsible for the maintenance and spread of rheumatoid synovitis (2). Inherent in this process is an active proliferation of small blood vessels necessary for the extensive emigration of mononuclear cells and proliferation of the synovial tissue characteristic of this type of synovitis. The blood vessels that participate in the synovial proliferation develop in a series of sequential steps in which endothelial cell (EC)<sup>1</sup> proliferation

is an absolute requirement (1). Inhibition of EC proliferation, therefore, would have the potential to slow or diminish the immunologically mediated inflammation of rheumatoid arthritis (RA). Because EC may participate in immunological reactions by presenting antigen to T and B lymphocytes (3–6) and secreting interleukin 1 (IL-1) (7, 8), a decrease in the EC population may also diminish synovial inflammation by affecting these processes.

Gold salts have been used in the treatment of RA for over 50 yr. They have been reported to not only relieve the symptoms of rheumatoid inflammation but also to slow the progress of the disease (9–12). Though a variety of actions of the gold salts have been reported to be responsible for their therapeutic efficacy (13), the mechanism underlying their beneficial effects has not been adequately elucidated. To investigate the possible effects of gold compounds on angiogenesis, we have studied the effects of gold sodium thiomalate (GST), gold chloride, and 2,3,4,6-tetra-*O*-acetyl-1-thio-D-glucopyranosato-*S*-(triethylphosphine) (auranofin) on basal and endothelial cell growth factor (ECGF)-stimulated proliferation of human umbilical vein EC. It has been shown that low concentrations of these gold compounds, easily attainable in the serum of treated patients, inhibit EC proliferation. Inhibition of fibroblast proliferation was observed in the same approximate concentration range.

## Methods

*Preparation of human EC and EC monolayers.* EC were obtained from human umbilical veins as described previously with some modifications (14). Fresh umbilical cord was placed in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY). The umbilical veins were cannulated and perfused with HBSS to wash out the residual blood. Both ends of the cord were clamped and then infused with 1% collagenase (Worthington Diagnostics Div., Millipore Corp., Freehold, NJ) in RPMI 1640 (Gibco) for 15 min at room temperature. The collagenase solution containing detached EC was flushed out of the cord with RPMI 1640. The cells were then centrifuged and resuspended in complete medium, i.e. RPMI 1640 containing 15% heat-inactivated fetal calf serum (FCS) (Gibco), 10% heat-inactivated human serum, 25  $\mu\text{g/ml}$  ECGF (Collaborative Research, Inc., Waltham, MA), 5 U/ml heparin, and antibiotics. EC were cultured overnight in 25  $\text{cm}^2$  tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 95% air). The next day, contaminating cells were vigorously rinsed from the flasks and fresh complete medium added.

When the primary cultures reached confluency, EC were trypsinized, resuspended in complete medium, and seeded into three gelatin-coated flasks for further passage. EC contained in this way were used in the third or fourth passage for the experiments described below. The identity of the EC was established by their characteristic morphology under phase-contrast microscopy and by indirect immunofluorescence with a rabbit anti-Factor VIII antiserum (Cappel Laboratories, Cochranville, Chester, PA). All cells were positively stained. The percent of cells staining positively with the antimacrophage antibody 63D3 (15) by FACS analysis was < 1%. All individual batches of EC were prepared from single umbilical cords.

EC were detached from confluent monolayers by trypsinization, and further cultures were carried out in triplicate in sterile, gelatin-coated,

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1. *Abbreviations used in this paper:* Con A, concanavalin A; EC, endothelial cell; ECGF, endothelial cell growth factor; GST, gold sodium thiomalate; IFN- $\gamma$ , gamma interferon; IL-1, interleukin 1; PHA, phytohemagglutinin; RA, rheumatoid arthritis; TdR, thymidine; TMA, thiomalic acid.

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flat-bottomed microtiter wells (6.4 mm diameter) (Corning Glass Works). Each well contained  $2 \times 10^4$  responding cells in 0.2 ml culture medium, consisting of RPMI 1640 containing 5% heat-inactivated FCS and antibiotics. This medium was used in all EC proliferation assays unless otherwise stated. The microtiter wells were then placed in a CO<sub>2</sub> incubator for 3–4 h to obtain EC monolayers.

**Assay for tritiated thymidine (3H-TdR) incorporation into EC.** Cultures were carried out in the presence or absence of various concentrations of ECGF or ECGF with 1 U/ml of sodium heparin (Upjohn Co., Kalamazoo, MI) for 48 h. 15 h before harvesting, 1  $\mu$ Ci of 3H-TdR was added to each well. At the end of the incubation period, EC were washed three times with phosphate-buffered saline (PBS) (0.05 M, pH 7.4) and detached from the microtiter wells by trypsinization. Detached EC were harvested onto glass fiber filter paper using a mini-MASH II microharvesting device (Whittaker M. A. Bioproducts, Walkersville, MD) and 3H-TdR incorporated into EC was determined using a liquid scintillation counter.

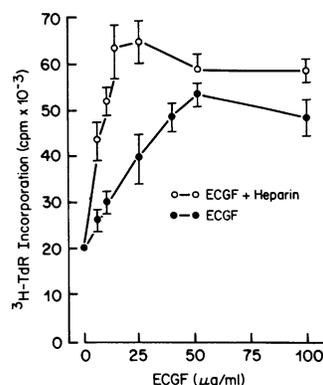
**Effects of gold compounds on 3H-TdR incorporation into EC.** Cultures were done in the presence or absence of ECGF or ECGF with 1 U/ml heparin (ECGF-heparin), and, simultaneously, with and without various concentrations of GST (Merck Sharp & Dohme Div., Merck & Co., Inc., Rahway, NJ), thiomalic acid (TMA) (Merck Sharp & Dohme), auranofin (Smith Kline & French Laboratories, Philadelphia, PA), or gold chloride (auric chloride or chloroauric acid, Sigma Chemical Co., St. Louis, MO). 3H-TdR incorporated into EC was counted as described above. To examine the time-dependent effects of GST and auranofin on the DNA synthesis of EC, these cells were cultured with ECGF-heparin in the presence of these agents for increasing time periods up to 48 h. The EC in the microtiter wells were then washed with culture medium to remove extracellular GST and auranofin. Fresh culture medium containing ECGF-heparin was then added to the wells and the cultures continued. Incorporation of 3H-TdR, added 15 h before harvesting, was measured 48 h after initiation of the cultures. To examine the effect of these gold compounds on any given phases of EC proliferation, they were added to the EC culture at the initiation of incubation or at varying times thereafter.

**Assay for 3H-TdR incorporation into human fibroblasts.** Primary cultures of fibroblasts were established from explants of human foreskins by standard technique. Fibroblasts in their fifth to tenth subpassage were harvested from stock cultures by trypsinization and suspended into RPMI 1640 supplemented with 10% FCS and antibiotics. 3H-TdR incorporation into the cells after stimulation with IL-1 was measured as described previously with slight modifications (16).  $1 \times 10^4$  responding cells in 0.2 ml culture medium were cultured in flat-bottomed microtiter wells (Corning Glass Works) and 2 U/ml ultrapure IL-1 (Genzyme Corp., Boston, MA) was added to the cultures in the presence or absence of varying concentrations of GST. The total time of the assay was 72 h. 1  $\mu$ Ci of 3H-TdR was added to the culture 15 h before harvesting and 3H-TdR incorporation was measured as described above.

**Assay for 3H-TdR incorporation into peripheral blood mononuclear cells (PBM).** PBM were obtained by centrifuging venous blood obtained from healthy human donors on Ficoll Hypaque (Lymphoprep, Nyegaard & Co., Oslo, NJ) as previously described (17). PBM were then suspended in RPMI 1640 containing 10% FCS and antibiotics.  $1 \times 10^5$  PBM, in 0.2 ml of the culture medium, were then placed in round-bottomed microtiter wells (Corning Glass Works). Cultures were then carried out in the presence of 5  $\mu$ g/ml concanavalin A (Con A, Sigma Chemical Co.) or 0.5  $\mu$ g/ml phytohemagglutinin (PHA, Sigma Chemical Co.) in the presence of varying concentrations of GST. 15 h before harvesting, 1  $\mu$ Ci of 3H-TdR was added to each well. Total duration of the cultures was 72 h. The cells were then harvested onto glass fiber filter paper and incorporated 3H-TdR counted as described above.

## Results

**Response of EC to ECGF and heparin.** To study the dose response of EC to ECGF, the monolayers derived from  $2 \times 10^4$



**Figure 1.** 3H-TdR incorporation in EC stimulated with ECGF alone or ECGF plus heparin.  $2 \times 10^4$  EC were stimulated with varying concentrations of ECGF or ECGF plus 1 U/ml heparin sodium, and after 48 h, 3H-TdR incorporation was measured. Each point represents the mean  $\pm$  SE of five separate experiments, each done in triplicate.

EC in microtiter wells were incubated with increasing concentrations of ECGF. Increase of 3H-TdR incorporation into EC occurred in a dose-dependent fashion (Fig. 1). Significant increase of 3H-TdR incorporation was detected at 6.25  $\mu$ g/ml ECGF ( $P < 0.01$ ). The increase was linear up to 50  $\mu$ g/ml and then leveled off. The response of EC to ECGF was enhanced in the presence of heparin (Fig. 1). As the effect of heparin reached maximal at a concentration of 1 U/ml (data not shown), this concentration was used for subsequent experiments. Whereas half-maximal response of 3H-TdR incorporation in the presence of ECGF alone occurred at a concentration of 25  $\mu$ g/ml, this occurred at a concentration of 6.25  $\mu$ g/ml in the presence of heparin. Heparin alone added to the culture medium suppressed 3H-TdR incorporation (Table I).

**Effect of GST on EC growth.** Addition of GST to EC cultures suppressed both ECGF and ECGF-heparin-induced 3H-TdR incorporation (Fig. 2). Dose response curves of EC growth to ECGF and ECGF-heparin in the presence of GST showed that this drug decreased the magnitude of the EC response to these stimuli in a concentration-dependent fashion. On the other hand, the dose of ECGF required for half-maximal response of the EC was not affected by GST.

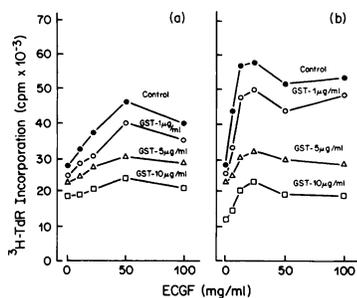
To exclude the possible effect of the serum present in the cultures on the action of GST, we added varying concentrations of FCS or human serum to the 3H-TdR incorporation assay. FCS, in the range 5–25%, did not significantly affect the magnitude of the inhibitory effect of GST on both basal and stimulant-induced DNA synthesis in EC, nor did 10% human serum added to 15% FCS have a significant effect (data not shown).

**Comparison of effects of GST on proliferation of EC, fibroblasts, and peripheral blood mononuclear cells.** To compare the

**Table I.** Effect of Heparin on Basal DNA Synthesis of EC

Concentration heparin	<sup>3</sup> H-TdR Incorporation	P Value*
U/ml	cpm	
0	24,670 $\pm$ 2,780	—
1	7,830 $\pm$ 1,290	< 0.01
5	5,340 $\pm$ 1,030	< 0.01
10	5,050 $\pm$ 1,220	< 0.01

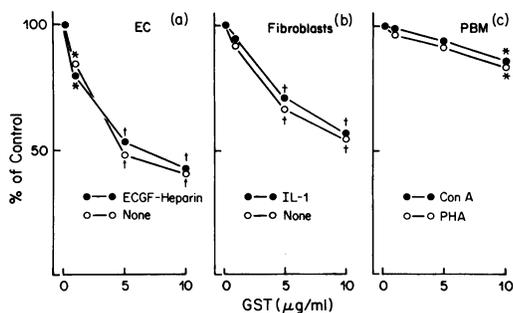
$2 \times 10^4$  EC were cultured with or without increasing concentrations of heparin, and after 48 h, 3H-TdR incorporation was measured. Each number represents the mean  $\pm$  SE of three separate experiments, each done in triplicate. \* Student's *t* test.



**Figure 2.** Effect of GST on dose response of EC proliferation to various concentrations of ECGF or ECGF-heparin. EC were cultured in microtiter wells with increasing concentrations of ECGF (a) or ECGF plus 1 U/ml heparin (b) and varying concentrations of GST added simultaneously.  $^3\text{H-TdR}$  incorporation was as-

sayed 48 h after the initiation of the culture. Each point represents the mean of eight separate experiments, each done in triplicate.

dose-dependent inhibitory effect of GST on stimulant-induced EC DNA synthesis with that of IL-1-induced fibroblast DNA synthesis and mitogen-triggered DNA synthesis in PBM, various concentrations of GST were added to these cultures. Both basal and ECGF-heparin-induced EC response was suppressed in a dose-dependent fashion, with significant inhibition observed in the presence of 1  $\mu\text{g/ml}$  GST (Fig. 3 A). A near maximal effect was attained in the range of 5–10  $\mu\text{g/ml}$ . These concentrations are attained in both blood and synovium of patients treated with GST (18–25). Culture of EC with up to 10  $\mu\text{g/ml}$  GST did not affect the viability of these cells, both ECGF stimulated and



**Figure 3.** Inhibitory effect of GST on DNA synthesis of EC, fibroblasts, and PBM. (a) EC were cultured with or without 12.5  $\mu\text{g/ml}$  ECGF plus 1 U/ml heparin and varying concentrations of GST added simultaneously. After 48 h,  $^3\text{H-TdR}$  incorporation was measured. Total incorporated  $^3\text{H-TdR}$  in EC cultured only with ECGF-heparin (100% value,  $52,348 \pm 3,425$  SE cpm) or without ECGF-heparin (100% value,  $22,576 \pm 2,378$  SE cpm) were taken as controls. Each point represents the mean of six separate experiments, each done in triplicate. (b)  $1 \times 10^4$  fibroblasts were cultured with or without 1 U/ml ultrapure IL-1 and the indicated concentrations of GST added simultaneously.  $^3\text{H-TdR}$  incorporation was assayed 72 h after initiation of the assay. Total  $^3\text{H-TdR}$  incorporated into fibroblasts cultured without IL-1 (100% value,  $16,280 \pm 2,310$  SE cpm) or with IL-1 (100% value,  $30,220 \pm 1,860$  SE cpm) were taken as controls. Each point represents the mean of 3 separate experiments, each done in triplicate. (c)  $1 \times 10^5$  PBM were cultured with 5  $\mu\text{g/ml}$  Con A or 0.5  $\mu\text{g/ml}$  PHA in the presence of increasing concentrations of GST. After 72 h,  $^3\text{H-TdR}$  incorporated was assayed. Total  $^3\text{H-TdR}$  incorporated into PBM cultured with Con A alone (100% value,  $55,780 \pm 4,986$  SE cpm) or with PHA alone (100% value,  $88,674 \pm 7,023$  SE cpm) were taken as controls.  $^3\text{H-TdR}$  incorporation into PBM without mitogen stimulation was  $1,256 \pm 413$  SE cpm. Each point represents the mean of four separate experiments, each done in triplicate. Student's *t* test of each point was performed by comparison with the control. \*  $P < 0.05$ ; †  $P < 0.01$ .

unstimulated, when examined by trypan blue exclusion (data not shown), even though the EC assumed a more slender appearance.

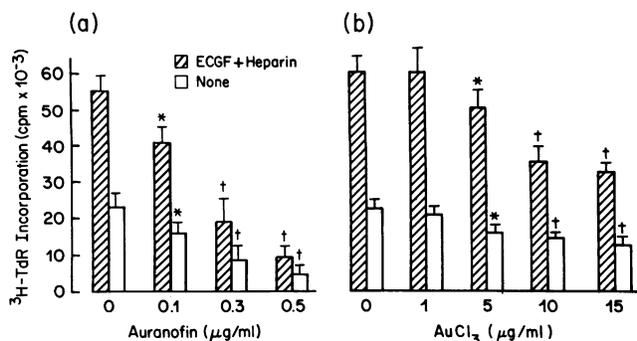
As fibroblasts, macrophages, and lymphocytes are constituents of rheumatoid synovium, the possible effects of GST on these cell types were examined. 5 and 10  $\mu\text{g/ml}$  GST significantly affected both basal and IL-1-triggered  $^3\text{H-TdR}$  incorporation into fibroblasts, though 1  $\mu\text{g/ml}$  manifested no effect (Fig. 3 B). In contrast, the inhibitory effect of GST on DNA synthesis of PBM activated by Con A or PHA was minimal within the ranges examined, with only 15% inhibition being observed at 10  $\mu\text{g/ml}$  and no significant effect at 1 or 5  $\mu\text{g/ml}$  GST (Fig. 3 C). When the effect of 10  $\mu\text{g/ml}$  GST on basal and stimulant-induced DNA synthesis of the three cell types is compared, it can be seen that the magnitude of inhibition of both basal and stimulant-induced DNA synthesis of EC and fibroblasts was similar. The degree of inhibition of DNA synthesis of these two cell types was significantly greater than that observed in Con A and PHA-stimulated PBM ( $P < 0.01$ ), indicating that EC and fibroblasts were more sensitive to GST than PBM. Regardless of the presence or absence of IL-1, PHA, or ConA, the concentrations of GST examined did not affect the viability of either the fibroblasts or PBM.

**Effect of TMA.** To rule out the possibility that the observed inhibitory effect of GST might be due to the free thiol of GST, we added TMA to the EC cultures in the same range of concentration as GST. TMA did not have a significant effect on either basal or ECGF-heparin EC responses in the range of concentrations in which GST had significant effects (data not shown).

**Effect of gold chloride and auranofin.** To determine whether other types of gold compounds have a similar effect on EC, we also examined gold chloride and auranofin for their effect on EC proliferation. The effect of various concentrations of gold chloride on  $^3\text{H-TdR}$  incorporation into unstimulated and stimulated EC is shown in Fig. 4 A. Significant inhibition ( $P < 0.05$ ) was observed at a concentration of 5  $\mu\text{g/ml}$  in both basal and ECGF-heparin-induced  $^3\text{H-TdR}$  incorporation with near maximal effect ( $P < 0.01$ ) in the range of 10–15  $\mu\text{g/ml}$ . These concentrations did not significantly affect the viability of the EC.

The effective dosage of auranofin was much lower than that of GST and gold chloride. As shown in Fig. 4 B, significant inhibition was observed at the level of 0.1  $\mu\text{g/ml}$ , and near-maximal effect was attained in the range of 0.3–0.5  $\mu\text{g/ml}$  auranofin. These concentrations are also attainable in both blood and synovium of patients treated with this drug. Incubation of EC with 0.5  $\mu\text{g/ml}$  auranofin did not significantly affect their viability.

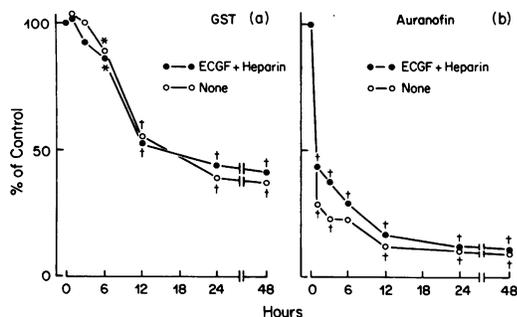
**Time-kinetic studies of effects of initial addition of GST and auranofin on EC DNA synthesis.** Results of time-kinetic studies of the effects of GST and auranofin on basal and ECGF-heparin-induced  $^3\text{H-TdR}$  incorporation in EC are shown in Fig. 5. EC were cultured with 10  $\mu\text{g/ml}$  GST or 0.5  $\mu\text{g/ml}$  auranofin for 0–48 h in the presence of ECGF and heparin. At varying time periods, the supernatant was removed and EC were washed and then cultured with or without fresh ECGF and heparin. When GST was present for as little as the initial 6 h of culture, significant inhibition of  $^3\text{H-TdR}$  incorporation was observed both in non-stimulated and stimulated EC (Fig. 5). Near-maximal inhibition was observed after 12 h. It appears, therefore, that the initial 6-h period is a critical time interval for the induction of the suppressive effect of GST and that this inhibition is not reversible within the subsequent assay period. In the case of auranofin, more than half-maximal suppression was observed in both basal



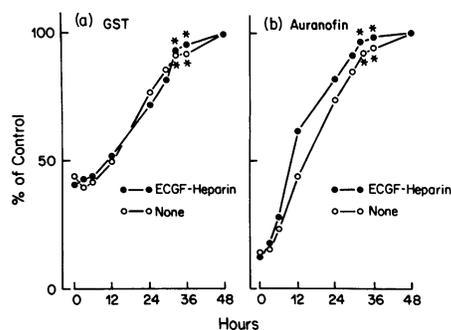
**Figure 4.** Effect of auranofin and gold chloride on DNA synthesis of EC. EC were cultured with or without 12.5 µg/ml ECGF plus 1 U/ml heparin in the presence of the indicated concentrations of (a) auranofin or (b) gold chloride. 3H-TdR incorporation was assayed after 48 h. Each bar represents the mean ± SE of five separate experiments, each done in triplicate. Student's *t* test has been done to compare experimental values with controls (\* *P* < 0.05; † *P* < 0.01).

and ECGF-heparin stimulated DNA synthesis following the presence of this agent for only the initial 1 h of culture (Fig. 5). Thus, the effect of auranofin on EC appeared to be more rapid in onset than that of GST.

**Effects of delayed addition of GST and auranofin on EC DNA synthesis.** DNA synthesis in the course of EC proliferation, as assayed by 3H-TdR incorporation, is a late occurrence in a series of reactions. To determine whether a particular sequential step was sensitive to inhibition by gold compounds, we added GST or auranofin to unstimulated and ECGF-heparin-stimulated EC cultures at the initiation of incubation or at varying times thereafter. Fig. 6 shows the effects of GST on basal and ECGF-heparin-induced 3H-TdR incorporation into EC. Significant inhibition was detected when GST was added at least 18 h before the end of the assay. GST added at the time of the



**Figure 5.** Time-dependent effect of initial addition of GST and auranofin on DNA synthesis of EC. EC were cultured with or without 12.5 µg/ml ECGF plus 1 U/ml heparin, and 10 µg/ml GST (a) or 0.5 µg/ml auranofin (b) were added for the indicated time intervals from the initiation of the assay. At the end of these intervals, the GST or auranofin was removed, the EC were washed and recultured with or without ECGF plus heparin until the termination of the assay and 3H-TdR incorporated was then measured. EC cultured with ECGF plus heparin (100% value, 60,463 ± 5,246 SE cpm) or without ECGF plus heparin (100% value, 27,586 ± 1,870 SE cpm) in the absence of GST or auranofin served as controls. Each point represents the mean of three separate experiments, each done in triplicate. Student's *t* test was used to compare experimental values with controls (\* *P* < 0.05; † *P* < 0.01). In these experiments, the concentration of ECGF, which produced half-maximal growth response in the presence of heparin, was 6.25 µg/ml.



**Figure 6.** Time-dependent effect of delayed addition of GST and auranofin on DNA synthesis of EC. EC were cultured in the presence or absence of 12.5 µg/ml ECGF plus 1 U/ml heparin, and 10 µg/ml GST (a) or 0.5 µg/ml auranofin (b) was added to the cultures at the initiation of incubation or at varying times thereafter. 3H-TdR incorporation was assayed after 48 h. EC cultured with ECGF plus heparin (100% value, 55,473 ± 4,976 SE cpm) or without ECGF plus heparin (100% value, 23,219 ± 3,126 SE cpm) in the absence of GST and auranofin were taken as controls. Each point represents the mean of three separate experiments, each done in triplicate. Experimental values were compared with controls by Student's *t* test. \* NS.

3H-TdR pulse, i.e., 15 h before termination of the assay or thereafter, did not have a significant effect on the subsequent incorporation of 3H-TdR. The latter observation indicates that GST did not influence the transport of the 3H-TdR into the cells.

Similar effects were observed following the delayed addition of auranofin. When the drug was added at the time of the 3H-TdR pulse or thereafter, no significant effect was observed (Fig. 6). The suppression of 3H-TdR incorporation following initial and delayed addition of GST or auranofin is compared in Fig. 6. The presence of GST during the initial 24 h inhibited 51% of the total 3H-TdR incorporation induced by ECGF with heparin, whereas the presence of GST during only the last 24 h induced only 29% inhibition, which was significantly smaller (*P* < 0.01). A similar result was observed in the case of basal DNA synthesis. Significantly greater suppression of DNA synthesis (*P* < 0.01) was also observed in the presence of auranofin during the initial 24 h than during the last 24-h culture.

## Discussion

Gold salts have been administered to slow or halt the progression of RA. Suggested mechanisms underlying the therapeutic efficacy of these drugs include the inhibition of lysosomal or other cellular enzymes (26–28), interference with complement activation (29, 30), inhibition of prostaglandin biosynthesis (31, 32), alterations in protein interactions (33–36) and sulfhydryl reactivity (37), and nonspecific antiinflammatory activity (38). It has also been suggested that they block the accessory cell functions of macrophages, leading to regression of cell-mediated immunological reactions (39). Some of these mechanisms, however, have been based on observations made with concentrations of gold salts not attained in serum of the treated patients. Moreover, they have not explained the delayed onset of the therapeutic effects of these agents.

The rheumatoid synovial membrane is characterized by extensive proliferation of the connective tissue and infiltration of this tissue by chronic inflammatory cells. Associated with these

two processes is an active proliferation of synovial small blood vessels or neovascularization. This process supplies the extensive network of venules, postcapillary venules, and capillaries from which inflammatory cells infiltrate the synovium and which, in addition, supports the fibroblast proliferation essential for the growth of the synovium and development of pannus. Therefore, interruption of neovascularization would be expected to interfere with mononuclear cell infiltration and synovial proliferation. It is for this reason that we have attempted to investigate the possible effects of gold compounds on neovascularization. Because vascular proliferation is critically dependent on local EC proliferation, the effects of gold compounds on *in vitro* proliferation of EC have been examined. Moreover, in contrast to other mononuclear cells such as macrophages, which are resupplied to inflammatory tissue from the bone marrow, there is no reservoir of EC to replenish diminished or insufficient numbers of EC, so it has been of particular interest to examine the effects of gold compounds on the growth of this cell type.

The present data demonstrate that GST inhibits DNA synthesis of EC both in a dose and time-dependent fashion. TMA, on the other hand, in the same range of concentration as GST, did not significantly affect EC proliferation, indicating that the observed effects depend upon the gold ion itself and not on the TMA residue. This conclusion is also supported by the observation that similar inhibition of DNA synthesis in EC cultures was exerted by gold chloride and auranofin.

Significant inhibitory effects of GST and auranofin on EC proliferation were observed at concentrations of 1  $\mu\text{g/ml}$  and 0.1  $\mu\text{g/ml}$  respectively. Maximal inhibition was obtained with 5–10  $\mu\text{g/ml}$  GST and 0.3–0.5  $\mu\text{g/ml}$  auranofin. Although the serum concentration of gold varies, depending upon the dosage administered and dosage schedule, it is generally accepted that serum levels attained in GST-treated patients tend to be in the range of 2–5  $\mu\text{g/ml}$  (18–23), which is equivalent to 4–10  $\mu\text{g/ml}$  GST. It has also been reported that the blood concentration range of auranofin is 0.3–1.0  $\mu\text{g/ml}$  (40). The levels of gold in the synovial tissue of long-term-treated patients are much higher, *i.e.*, 21–25  $\mu\text{g/g}$  of tissue, wet wt (24, 25), equivalent to 42–50  $\mu\text{g/ml}$  GST although much of this gold may be sequestered in a relatively small number of phagocytic cells. The concentrations of GST and auranofin, observed to suppress the proliferation of EC in the present experiments are, therefore, easily attainable in the serum and synovium of treated patients. The effective concentrations required for the inhibition of EC proliferation were also 10% of those required for the inhibition of lymphocyte DNA synthesis. The present observations, therefore, support the possibility that an inhibitory action of gold compounds on vascular proliferation may be the critical event that leads to clinical improvement in patients treated with these drugs. Since the numbers of EC present in a tissue are determined by local proliferation and are not replenished from a distant source, it is possible that the characteristic delay in the onset of the therapeutic effect of gold compounds may represent the time required for the EC population to fall below the critical level needed to support the ongoing inflammatory and proliferative processes.

Fibroblasts have been reported to increase their proliferation in response to cytokine-rich supernatants of PBM (41) or IL-1 (16, 42). We have also observed in these experiments that GST, in the concentrations used, was able to inhibit both unstimulated and IL-1-stimulated fibroblast proliferation. Inhibition of this cell type was also produced by gold chloride and auranofin in the range of concentrations required for significant suppression

of EC proliferation (data not shown). This observation is pertinent to the effect of gold compounds on rheumatoid synovitis because proliferation of fibroblasts and fibrosis are central features of the growth of the rheumatoid pannus. It is possible, therefore, that gold compounds may decrease the growth of pannus by also inhibiting local fibroblast proliferation.

Numerous factors have been reported to support the process of neovascularization. Heparin, a product of mast cells that can bind ECGF (43, 44), has been found to enhance the affinity of this agent for its EC surface receptor (45). Leukocytic infiltration has been observed to precede vascularization in the cornea (46), and agents present in supernatants of cultured macrophages (47) and activated macrophages themselves have been observed to stimulate neovascularization (48). IFN- $\gamma$ , produced by activated T cells and IL-1, a product of macrophages, have also been suggested to play a role in the induction of angiogenesis by increasing the release of superoxide from EC (49). Finally, fibronectin, which is secreted by fibroblasts, has been observed to be a chemoattractant for EC (50). These observations suggest that products of chronic inflammatory cells and fibroblasts may be intimately associated with angiogenesis in chronically inflamed tissue such as rheumatoid synovium. They are also consistent with the finding that increased vascularity is observed in the transitional areas of RA synovium, in which a mixture of interacting cell types (lymphocytes, macrophages, fibroblasts, and plasma cells) is present (51).

The mechanism by which gold compounds inhibit EC proliferation is unresolved at the present time. It is unlikely that the observed effects of GST are due to interference with the binding of ECGF to EC or with the action of heparin in the binding of ECGF to EC. This is suggested by the finding that GST, present only in the initial 1–3-h incubation period, had little effect on the magnitude of the resultant DNA synthesis induced by both ECGF alone and ECGF with heparin. Moreover, the observations that GST also blocked 3H-TdR incorporation into nonstimulated EC and that the concentration of ECGF required to achieve a half-maximal response of EC to the stimulation of ECGF or ECGF plus heparin was not affected by GST are also consistent with this suggestion. The observation that GST had a much stronger inhibitory effect on DNA synthesis when present during the initial 24 h of culture than in the last 24 h indicates that GST may affect a comparatively early step in the process of EC proliferation.

GST has been suggested to bind sulfhydryl-containing compounds on the basis that its action on lysosomal hydrolases is blocked by cysteine (19) and its action on lymphocyte DNA synthesis is inhibited by D-penicillamine (52). Preliminary experiments in this laboratory have also shown that D-penicillamine is capable of interfering with the GST-induced suppression of DNA synthesis of EC. It is therefore conceivable that interaction of GST with EC-associated sulfhydryl-containing compounds may be involved.

In conclusion, GST and auranofin inhibited both basal and ECGF-induced DNA synthesis in human EC. These effects of gold compounds may play a significant role in the suppression of rheumatoid inflammation, because a decrease in the number of local blood vessels may lead to diminished mononuclear cell infiltration and decreased proliferation of the synovial tissue. Such a decrease may also interfere with the level of accessory cell activity on the part of the EC at the blood tissue interface. Because EC are not replenished from other sources, the delay in onset of the therapeutic effect of gold compounds may rep-

resent the time required for reducing the size of the vascular bed in synovial inflammatory foci below a critical level.

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