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THE EFFECTS OF RAZOXANE (ICRF 159) ON THE PRODUCTION OF COLLAGENASE AND INHIBITOR (TIMP) BY STIMULATED RABBIT ARTICULAR CHONDROCYTES

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Abstract—Monolayer cultures of rabbit chondrocytes were stimulated to produce collagenase with conditioned medium from human peripheral blood mononuclear cells (MCM), and the ability of Razoxane to modulate the production of collagenase and specific tissue inhibitor of metalloproteinases (TIMP) was studied. Collagenase production was inhibited and TIMP increased by Razoxane, in a dose-dependent manner, when cells were treated daily for 3 days. Over this period the effect of Razoxane was progressive; 50 μg/ml or less had no effect at day 1 but 50 μg/ml was effective by day 3. The effectiveness of Razoxane was inversely related to the degree of MCM stimulation and the confluency of the culture. On removal of the drug, chondrocytes stimulated with MCM recovered their ability to produce collagenase, and TIMP production returned to near normal. The results suggest that the ability of Razoxane to reduce collagenase and increase TIMP production may correlate with its effectiveness in treating psoriatic arthritis.

The chondrocytes and synovial fibroblasts of the arthritic joint have been shown to have the capacity to resorb their own matrix [1, 2]. In culture, these cells produce neutral metalloproteinases [3–6] and TIMP† [7], whereas normal chondrocytes and synovial cells produce TIMP but only small or undetectable amounts of metalloproteinases. Factors derived from blood mononuclear cells have been shown to stimulate metalloproteinase production by cells from both normal and arthritic joints [3, 6, 8–11]. It has been proposed that *in vivo* TIMP is important in controlling the local activity of the enzymes to prevent resorption; in the arthritic joint, stimuli such as blood mononuclear cell factors could cause a local excess of enzyme over inhibitor and allow resorption to take place [12]. In support of such ideas increases in collagenase and reductions in TIMP production by synovial and cartilage explants from rabbits with an experimentally-induced arthritis have been found to correlate with the severity of the lesion [13, 14]. Consequently control over the release of metalloproteinases and their inhibitors is of considerable interest from a therapeutic standpoint. The steroidal anti-inflammatory drugs have been shown to inhibit production of metalloproteinases in culture [11, 15–17], but the non-steroidal anti-rheumatic drugs generally prevent metalloproteinase release from cultured cells only at cytotoxic doses [11, 18].

Razoxane [(±)-1,2-bis(3,5-dioxopiperazin-1-yl)-propane; ICRF 159] has been used effectively in the treatment of severe cutaneous and arthropathic psoriasis [19, 20]. This derivative of EDTA was originally synthesized as an antitumour drug [21], after the observation that most useful antitumour drugs were either actual or potential chelating agents [22]. EDTA is ineffective against experimental tumours; this lack of activity is thought to be due to its highly polar structure which would prevent adequate absorption by the cell [23]. Razoxane is non-polar, and has been shown to penetrate cells and be hydrolysed to a chelating species which is itself too polar to enter the cell [24]. The drug delays progression through the cell cycle, an effect which is reversible on removal of the drug and which is independent of its cytotoxic action. The drug must be present just before mitosis to reduce viability markedly but the role of chelation in this process is uncertain [24]. The rationale for the use of Razoxane in the treatment of psoriasis was based on the similarity between the microvasculature seen in solid malignant tumours and that associated with rapid epidermal proliferation in psoriasis; Razoxane has been shown to 'normalize' the microvasculature in primary tumours [25].

In the present study we aimed to determine whether the effectiveness of Razoxane in psoriatic arthritis might be correlated with an ability of the drug to reduce metalloproteinase production by cartilage *in vitro*. Cultured chondrocytes provide a convenient *in vitro* assay for potential anti-arthritis drugs, since they can be induced to produce metalloproteinases by the addition of conditioned medium from mononuclear cells stimulated with lectins [3, 6, 12]. We show that Razoxane can inhibit the pro-

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† Abbreviations: TIMP, specific tissue inhibitor of metalloproteinases; PBS, phosphate-buffered saline; DMEM, Dulbecco's minimal essential medium; NIRS, non-inhibitory rabbit serum; Con A, concanavalin A; MCM, mononuclear cell conditioned medium; DMSO, dimethyl sulphoxide.

duction of collagenase, a marker enzyme for metalloproteinases in general.

MATERIALS AND METHODS

Mononuclear cell conditioned medium. Human mononuclear cells from heparinized peripheral blood were separated on Ficoll-paque (Pharmacia) according to the method of Boyum [26]. Cells were washed with PBS and plated at 4×10^6 cells/3 cm diameter dish in 2 ml DMEM (Gibco) supplemented with 58.4 μg/ml glutamine, penicillin/streptomycin (300 and 500 μg/ml, respectively) and 10% NIRS [27], plus 100 μg/ml Con A (Sigma). The cells were incubated at 37° for 48 hr in an atmosphere of 5% CO_2 in air. The medium, MCM, was then harvested at 900 *g* for 10 min and stored at −20° until use.

Chondrocyte cell culture. Chondrocytes from 10-day-old rabbits were prepared as described previously [6, 11]. The cells were used at first passage; on reaching confluence, the monolayer was washed three times with PBS and 1 ml DMEM supplemented as above and 10% NIRS were added, with or without the drug or factor under study. Treatments were

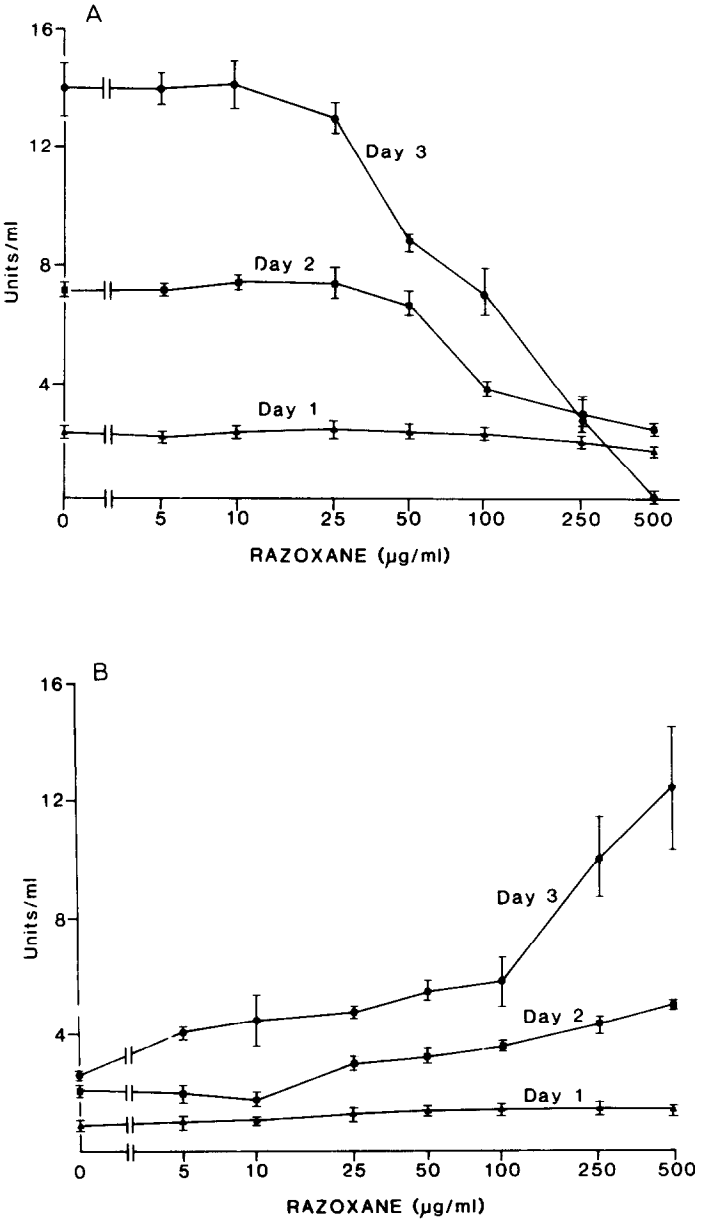

Fig. 1. The daily production of collagenase and TIMP by chondrocytes. Cells were stimulated with 10% MCM and treated for 3 days with varying concentrations of Razoxane; ▲, day 1; ■, day 2; ●, day 3. Results are expressed as units/ml of culture medium ±S.E.M. ($n = 4$) and the doses are on a logarithmic scale; (A) Collagenase; (B) TIMP.

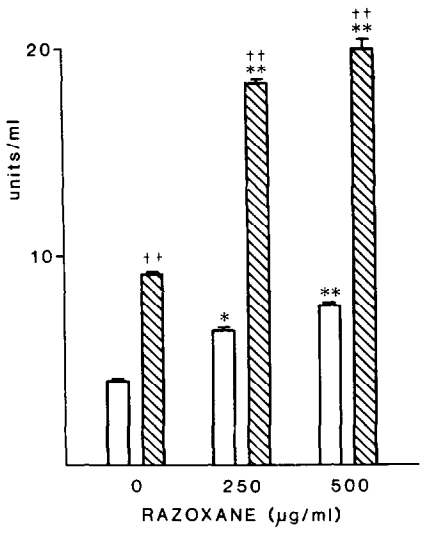

Fig. 2. Three-day accumulative levels of TIMP produced by chondrocytes treated with varying concentrations of Razoxane, in the presence and absence of MCM. Results are expressed as units/ml of culture medium ±S.E.M. ($n = 4$). □, No MCM; ▨, 10% MCM. *$P < 0.05$, **$P < 0.01$ comparing Razoxane treatment with untreated controls; ++ $P < 0.01$ comparing MCM stimulated with unstimulated.

normally carried out for 3 days; the supernatant was collected and fresh medium, with the appropriate supplements, was added to the cells every 24 hr. At the end of the experiment, cell viability was assessed by trypan-blue staining of the monolayer.

Drugs. Razoxane (kindly provided by ICI) was dissolved in 0.4 N HCl at 7.5 mg/100 μl, then diluted in medium and filter-sterilized. ICRF 198 (kindly provided by Dr. Creighton, Imperial Cancer Research Fund) was dissolved in DMSO at 125 μg/5 μl and diluted in medium, without further processing. Fresh solutions of either Razoxane or ICRF 198 were made before each addition to cells. Control cultures were always included in experiments, containing appropriate volumes of either 0.4 N HCl or DMSO without drug. DMSO concentrations never exceeded 0.5%. In the standard fibril assay for collagenase, Razoxane and ICRF 198 were dissolved and diluted as above; controls containing medium with or without appropriate volumes of either DMSO or 0.4 N HCl were included in each assay.

Assays. Media were assayed for collagenase and TIMP activities using ^{14}C-acetylated collagen fibrils as substrate. Total collagenase was assayed in the presence of para-aminophenyl mercuric acetate (0.3 nmole/ml) as an activator of latent enzyme. One unit of collagenase hydrolyses 1 μg of collagen/min at 35°. One unit of TIMP blocks the activity of 2 units of collagenase by 50% [27].

Statistical analysis. All results are expressed as the mean ± standard error of the mean, and comparisons were analysed by the two-tailed Student's *t*-test for significance.

RESULTS

The effect of Razoxane on MCM-stimulated production of collagenase by chondrocytes *in vitro* is shown in Fig. 1A. Razoxane inhibited collagenase production and also raised TIMP production (Fig. 1B) in a dose-dependent manner, and over the 3

Table 1. The effect of Razoxane on collagenase production by chondrocytes stimulated with high or low doses of MCM

%MCM	Razoxane (250 μg/ml)	Units/ml of collagenase in culture medium Day 1	Day 2	Day 3
5	−	0.99 ± 0.06	2.19 ± 0.09	3.46 ± 0.22
5	+	0.69 ± 0.05*	0.39 ± 0.05**	0**
20	−	3.50 ± 0.24	6.40 ± 0.38	7.74 ± 0.59
20	+	3.00 ± 0.25	5.43 ± 0.11	6.24 ± 0.53

Each result is the mean of four cultures ±S.E.M.
* $P < 0.05$, ** $P < 0.01$ comparing Razoxane treatment with untreated controls.

Table 2. The effect of Razoxane on collagenase and TIMP production by chondrocytes stimulated with MCM either at confluence or during cell growth

% confluency of cell monolayer at start of treatment	Razoxane 500 μg/ml	Units/ml in culture medium ±S.E.M. ($n = 4$) Collagenase Day 1	Collagenase Day 2	Collagenase Day 3	TIMP Day 3
100	−	2.81 ± 0.29	4.66 ± 0.47	11.90 ± 0.38	9.47 ± 0.90
100	+	1.73 ± 0.05	1.96 ± 0.09	0	14.96 ± 0.95
60	−	1.71 ± 0.09	4.47 ± 0.44	10.09 ± 0.32	9.10 ± 0.63
60	+	0.28 ± 0.01	0	0	17.83 ± 0.25

Cells were stimulated with 10% MCM and treated for 3 days, with or without 500 μg/ml Razoxane. Each result is the mean of four cultures ±S.E.M.
* $P < 0.05$, ** $P < 0.01$ comparing Razoxane treatment with untreated controls.

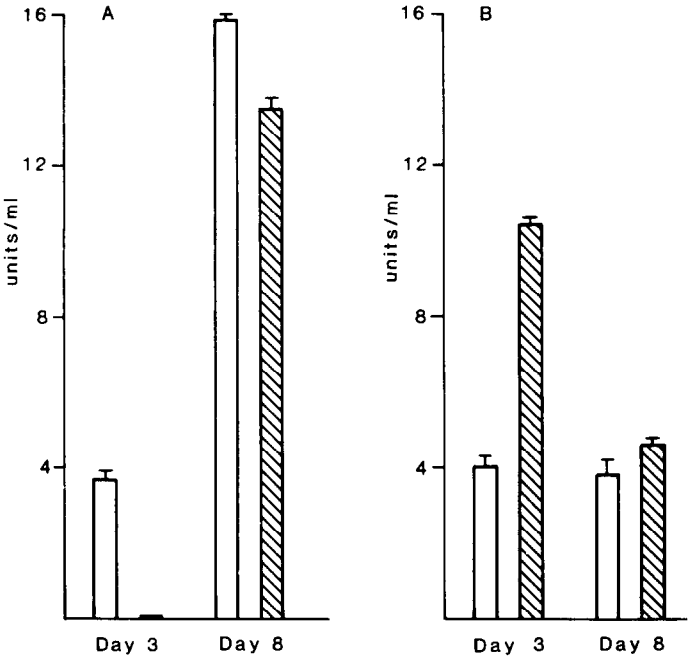

Fig. 3. Recovery of collagenase production by MCM-stimulated chondrocytes on the removal of Razoxane. Confluent monolayers of chondrocytes were treated from day 0 to day 3 with 5% MCM, with or without 250 μg/ml Razoxane. Razoxane treatment was terminated at day 3, but MCM stimulation continued daily to day 8. Results are expressed as the number of collagenase units (A) or TIMP units (B) per ml of culture medium ±S.E.M. ($n = 4$). □, Without Razoxane; ▨, 250 μg/ml Razoxane. *$P < 0.05$, **$P < 0.01$ comparing Razoxane treatment with untreated controls.

days of the experiment the effect of Razoxane was progressive. No dose was effective at day 1, but 100 μg/ml significantly reduced collagenase at day 2 ($P < 0.01$), and 50 μg/ml by day 3 ($P < 0.01$). Razoxane raised TIMP levels when added to chondrocytes not stimulated with MCM (Fig. 2). It can also be seen from Fig. 2 that MCM alone induces a rise in TIMP levels. Addition of cycloheximide at 1 μg/ml to Razoxane and MCM-treated cultures abolished collagenase and TIMP in the medium (results not shown), strongly suggesting that the changes in levels were due to changes in synthesis. Indomethacin (5 μg/ml, results not shown), a known inhibitor of prostaglandin synthesis, did not alter the effect of Razoxane on MCM-stimulated chondrocytes.

The effectiveness of Razoxane in decreasing collagenase production by chondrocytes was inversely related to the degree of MCM stimulation (Table 1). Razoxane at 250 μg/ml completely inhibited collagenase production by chondrocytes stimulated with 5% MCM, whereas the same dose had no significant effect on chondrocytes stimulated with 20% MCM. Razoxane was more effective at a given dose on cells which were dividing when treatment was initiated (Table 2). Cells which were 60% confluent at the start of treatment with MCM and Razoxane were more sensitive than were cells confluent at the initiation of treatment; collagenase production was completely inhibited by day 2 in subconfluent cells, but not until day 3 with confluent cells. This may reflect the difference in Razoxane concentration per cell in the two sets of monolayers. At day 3, when the subconfluent monolayer was approaching confluence, the levels of TIMP in the medium from cells treated with Razoxane and MCM were greater than in the medium from treated confluent monolayers. At this stage both sets of cells, treated with MCM only, produced similar concentrations of collagenase. As judged by trypan blue exclusion, Razoxane was not cytotoxic to either set of cells. In the subconfluent monolayer, a few cells were observed with enlarged nuclei, similar to those reported in other cell systems [24]. Although no definitive measurement was made, Razoxane did not appear to stop cell division, merely to delay it; a similar effect has been reported previously [24]. On removal of Razoxane after 3 days' treatment, MCM-stimulated chondrocytes recovered their ability to produce collagenase, and TIMP levels in the medium returned to nearly normal ($P > 0.05$, comparing Razoxane treated with untreated) after 5 days (Fig 3, A and B).

Preliminary experiments showed that ICRF 198, the hydrolysed chelating form of Razoxane [28], had no significant effects on either collagenase or TIMP production when added to MCM-stimulated chondrocytes (results not shown). Razoxane and ICRF 198 were also compared with EDTA for their ability to inhibit collagenase in the standard fibril assay (Table 3). Razoxane and ICRF 198 both inhibited collagenase, but not nearly as efficiently as EDTA. At 5 mM concentration, Razoxane inhibited lysis by only 11% compared to 22% for ICRF 198 or 66% for EDTA; below 5 mM Razoxane showed no inhi-

Table 3. Inhibition of collagenase action in fibril assays by EDTA, Razoxane and ICRF 198

Addition to reaction mixture	Collagenase, % residual activity
None	100
EDTA 5 mM	34
EDTA 10 mM	6
Razoxane 5 mM	89
Razoxane 10 mM	57
Razoxane 20 mM	30
ICRF 198 5 mM	78
ICRF 198 10 mM	44

Reaction mixtures were incubated for 18 hr at 35° in the standard fibril assay (see Materials and Methods). Collagenase activity is expressed as percentage of control level (48% fibril lysis with a standard collagenase preparation).

bition of lysis. Even at a 20 mM concentration Razoxane inhibited lysis by only 70%. In cell culture experiments even the highest dose of Razoxane used (500 μg/ml, 1.87 mM or less) was far lower than an effective inhibitory concentration in the collagenase fibril assay.

DISCUSSION

We have shown that Razoxane inhibits collagenase production by MCM-stimulated chondrocytes, and also increases the levels of TIMP found in the culture medium of the cells. Exposure of the chondrocytes to concentrations of 50 μg/ml (added daily) and above was effective after 3 days; this effect was shown to be progressive, and either longer treatment with the drug or more frequent additions would probably result in lower doses reducing collagenase production. In addition to the length of treatment, the effectiveness of a given dose of the drug was also shown to depend on the degree of MCM stimulation and the stage of confluency of the cell monolayer. These results illustrate that care is needed in interpreting the potency of drugs in cell culture systems.

Our data are similar to results using steroidal anti-inflammatory drugs [11, 15, 16], although inhibition of collagenase production by non-steroidal anti-inflammatory drugs is normally only seen at cytotoxic doses [11, 17]. At the doses tested in this paper, the drug was not cytotoxic, as judged by trypan blue exclusion, even in cultures of dividing cells. A slowing of cell division was observed, an effect similar to that reported by Creighton [24], albeit at lower doses (12.5 μg/ml or less). Such doses have been shown to be cytotoxic for BHK-21S and mouse L cells [24, 29], but only if present just before the 'G2/M border' of the cell cycle [24]; thus cytotoxicity would not be expected in non-dividing confluent monolayers. As the drug is excluded from cells by hydrolysis in the medium, the number of cells exposed to high doses at the sensitive period in a dividing culture will be relatively small if the drug is only added only at widely-spaced intervals and particularly if it also slows progression through the cell cycle.

The mode of action of Razoxane in reducing collagenase production and increasing TIMP by chondrocytes is unclear from these experiments. Razoxane has been shown to enter cells passively and be hydrolysed to a polar chelating species [23]; it is possible that intracellular chelation is partly or wholly responsible for the reduction in collagenase levels observed. ICRF 198, the hydrolysed chelating form of Razoxane, is excluded from cells by its polarity, and had no effects on collagenase and TIMP in MCM-stimulated chondrocyte medium; thus, extracellular chelation would not appear to be important in reducing collagenase levels in the culture medium. We have shown that Razoxane and ICRF 198 directly inhibit lysis of collagen fibrils by collagenase, presumably by chelation with Razoxane being hydrolysed to the active form during the 18 hr incubation period, although both compounds seem to be very poor inhibitors of the enzyme. The cumulative effect of Razoxane might suggest that it is trapped intracellularly, and hydrolysis to a polar species within the cell would prevent its egress: Dawson [23] found no evidence for physical or covalent binding to cellular macromolecules. The observation that collagenase and TIMP levels returned to normal on removal of the drug suggests that it may be metabolized in the cell. Further experiments are obviously needed to elucidate the mechanism of action of Razoxane in this cell system.

We consider that the action of metalloproteinases is important in the development of the arthritic lesion, and TIMP may regulate such activity under normal physiological conditions and prevent excessive extracellular degradation. Our results suggest that the effectiveness of Razoxane in treating psoriatic arthritis may correlate with an ability to reduce collagenase production in the joint, and Razoxane may offer an additional protection within the joint by raising production of TIMP. Razoxane has not proved to be as cytotoxic in our system as apparently in others [24, 29]; we are currently investigating analogues which are reportedly less cytotoxic and better tolerated *in vivo* to see whether they are as effective in reducing collagenase levels and to elucidate the mode of action and therapeutic effects of such compounds.

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