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Effect of Adenosine Analogues on the Expression of Matrix Metalloproteinases and Their Inhibitors From Human Dermal Fibroblasts

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ABSTRACT. The effect of the cytostatic and antiviral adenosine analogues 3-deazaadenosine (c^3Ado) and 3-deaza-(±)-aristeromycin (c^3Ari) on human skin fibroblasts was studied. Variables examined were cell morphology, viability, DNA fragmentation, expression of matrix metalloproteinases (MMPs) and matrix metalloproteinase inhibitors (TIMPs). None of these variables were changed when cells were exposed to c^3Ari concentrations ranging from 10^{-5} to 10^{-3} M or 10^{-5} M c^3Ado. However, large changes in cell morphology, viability and expression of MMPs and MMP inhibitors occurred when fibroblasts were treated with 10^{-4} or 10^{-3} M c^3Ado. Cells rounded up, shrank in volume, some detached and viability was lost without any detectable fragmentation of DNA. These changes in morphology and viability were associated with a differentiated expression of MMPs and MMP inhibitors. A large increase in collagenase activity occurred, and depending on the concentration of the adenosine analogue and the length of treatment, this change in activity could be shown to be due to one or a combination of the following factors: an increased synthesis of the collagenase protein, a decreased production of TIMP-1 or an increased activity of the collagenase superactivator, stromelysin. In contrast to this, treatment with c^3Ado resulted in a decreased gelatinase activity, which in part could be attributed to an increased production of an inhibitor that seemed to affect gelatinase but not collagenase. The cellular changes induced by c^3Ado seemed to reflect some of the alteration in the metabolic machinery that appears during a drug-induced or programmed/controlled death of a dermal cell. The different effects exerted by these two adenosine analogues on dermal fibroblasts can at least in part explain why c^3Ado have previously been shown to be more toxic than c^3Ari in animal models. BIOCHEM PHARMACOL **53**;10:1511–1520, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. adenosine analogues, matrix metalloproteinases, TIMP, cell morphology, cell viability

Many cell types synthesize matrix metalloproteinases (MMPs), a group of zinc- and calcium-dependent metalloenzymes which are inhibited by chelating agents. They are produced as proenzymes and can be activated *in vitro* by a variety of compounds including organomercurials, chaotropic agents, and proteases such as trypsin, chymosin, kallikrein, plasmin and cathepsin G [1–3]. MMPs cleave most, if not all, of the constituents of the extracellular matrix and the basement membrane [1–3]. A fine-tuned regulation of collagen turnover is essential for normal growth and development, wound healing, and the maintenance of normal tissue architecture in multicellular animals. A central control point in collagen metabolism is the enzyme that initiates the degradation of interstitial collagen, i.e. collagenase (MMP-1) [4]. Regulation of collagenase activity can occur at different levels: gene expression including transcription [5] and translation [6], activation and superactivation of the synthezised proenzyme, and inhibition of enzyme activity by tissue inhibitors of matrix metalloproteinases (TIMPs) [1–3]. Stromelysin (MMP-3) is a superactivator of collagenase but not the 72kDa gelatinase (MMP-2), i.e. it can act in synergy with other activators such as trypsin or p-aminophenylmercuric acetate (APMA) [1–3]. Hormones, growth factors and cytokines seem to play an essential role in modulation of MMP gene expression. MMP-1 and MMP-3 are coregulated by many compounds due to large similarities in their promotor regions [1–3]. However, MMP-2 is regulated differently compared to MMP-1 and MMP-3, due to different transcription factor motives in its promotor [1–3]. Compounds known to upregulate collagenase and stromelysin are phorbol esters, epidermal growth factor (EGF), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) while glucocorticoids, transforming growth factor-β (TGF-β) and interferon-γ (INF-γ) downregulate their expression [1–3].

MMPs play a role in normal remodeling events such as

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Abbreviations: MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of MMPs; c^3Ado, 3-deazaadenosine; c^3Ari, 3-deaza-(±)-aristeromycin; DMEM, Dulbecco's Modified Eagle's Media; APMA, p-Aminophenylmercuric acetate.

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blastocyst implantation, mammary gland involution after lactation and wound healing as well as in different pathological conditions [1–3]. In some cases, an altered level of different MMPs has been found to be associated with diseases such as epidermolysis bullosa [7–9], rheumatoid arthritis [1–3, 10], the development of metastasis in certain types of cancer [11, 12], systemic sclerosis and scleroderma [13, 14]. A search for compounds that could either modulate MMP expression at the cellular level or serve as direct inhibitors of these enzymes seems to be of importance in the development of pharmacological agents for different diseases. In this connection, the MMP inhibitor BB-94 (Batimastat) has been shown to inhibit human colon tumor growth and spread in a patient-like orthotopic model in nude mice [15], while some anthracycline antibiotics have been shown to inhibit collagen IV-degrading activity [16]. Likewise, some drugs used in therapy of the skin diseases epidermolysis bullosa [17] and in rheumatic diseases have been shown to influence MMP expression and activity [18].

3-deazaadenosine (c^3Ado) and 3-deaza-(±)-aristeromycin (c^3Ari) are adenosine analogues with diverse biological effects, probably mediated via interaction with multiple cellular targets [19–21]. Both compounds are inhibitors of S-adenosylhomocysteine hydrolase (AdoHcyase, EC 3.3.1.1), but only c^3Ado is a substrate for the enzyme [22]. These compounds exhibit similar broad activity against a variety of DNA and RNA viruses [22–24] and are assumed to have a therapeutic potential as anti-HIV-1 drugs acting on azidothymidine (AZT)-resistant strains [25]. c^3Ado, which is less selective and more toxic than c^3Ari [26], is also regarded as a potent immunosuppressive and anti-inflammatory agent [27, 28]. c^3Ari arrested HL-60 cells in the G2 phase without induction of apoptosis, while c^3Ado induced apoptosis in different phases of the cell cycle, depending on its concentration [19–21, 29]. The latter compound also induced disorganization of the microfilament system in mouse macrophages [30]. It was previously shown that microtubule disruptive agents which arrested cells in the methaphase could stimulate the production of MMPs in different cell types [31, 32]. As c^3Ado and c^3Ari are potential anti-viral agents that differ in cytotoxicity in animal models and in cancer cell lines, the intention of the present work is to clarify their effects on human skin fibroblasts. Tests were performed to investigate whether different concentrations of these two adenosine analogues were able to induce changes in cell morphology, viability, DNA fragmentation, MMP and MMP inhibitor synthesis.

MATERIALS AND METHODS

Chemicals

c^3Ado and c^3Ari were obtained from Southern Research Institute (Birmingham, AL, U.S.A.) Bovine serum albumin (BSA), trypsin, soyabean trypsin inhibitor (SBTI), acid-soluble calf skin and rattail collagens were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ELISA kits that specificially recognize total human TIMP-1 and MMP-1 were obtained from Amersham (Buckinghamshire, U.K.), the manufacturer codes being are RPN 2611 and 2610, respectively.

Cell Cultures

Human skin fibroblasts were maintained in Dulbecco's Modified Eagle's Media (DMEM) with high glucose, supplemented with 10% bovine calf serum, glutamine, non-essential amino acids, 5 mM Hepes buffer pH 7.2–7.4, streptomycin (100IU/mL) and penicillin (100μg/mL). Cells were kept in a humidified 5% CO_2 atmosphere at 37°C.

Cells (2×10^5 per well) were seeded on cluster six plates in DMEM containing 10% fetal calf serum and allowed to settle for approximately 36 hr. Thereafter, the cell layers were washed three times in Hanks Balanced Salt Solution (HBSS) and incubated in 2 mL serum-free DMEM with or without adenosine analogues. Both c^3Ado and c^3Ari were dissolved in serum-free medium and used at final concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. Control cells were incubated with the same volume of serum-free medium as drug-treated cells except for the omission of the drugs. Incubation times varied from 0 to 74 hr before the medium was harvested and assayed for immunoreactive collagenase, immunoreactive TIMP-1, MMP or MMP inhibitory activities. Half of the harvested serum-free medium was made 0.2% (w/v) in BSA/100 mM in Hepes buffer (pH 7.5)/10 mM in $CaCl_2$ (gelatinase and stromelysin) and the other was made 100 mM in Hepes buffer (pH 7.5)/10 mM in $CaCl_2$ (collagenase) [33].

The viability of cells untreated or treated with the adenosine analogues was investigated. After 74 hr incubation in serum-free medium, cells were washed three times in PBS, trypsinized, resuspended in DMEM supplemented with 10% serum plus additives, and counted in a Bürker chamber (Superior, Germany). Trypan blue exclusion was used to judge cell viability and membrane damage. Thereafter, 1×10^5 cells (treated and untreated) were seeded in T_{25} flasks, and serum containing DMEM was supplied every second day. At convenient time points, cells were trypsinized and counted.

Enzyme and Enzyme Inhibitor Assays

Collagenase, gelatinase and stromelysin were synthezised as proenzymes and activated with trypsin [33]. These enzymes were determined by measuring the trypsin-activated cell-conditioned medium's ability to degrade reconstituted [^{14}C]-collagen fibrils, [^{3}H]-gelatin and azocasein as previously described [33]. To determine whether any of the enzymes were produced in an active form, i.e. preactivated, enzyme activity was also measured on conditioned medium without trypsin activation. To determine the amount of MMP inhibitors in cell-conditioned medium, a large batch of fibroblast-conditioned medium was activated with trypsin for either 15 or 120 min and used in collagenase and

gelatinase assays, respectively. Aliquots of activated medium were mixed with increasing concentrations of unactivated cell-conditioned medium to a final volume of 100 μL (to measure collagenase activity) and 200 μL (to measure gelatinase activity) [9, 33]. MMP activity was determined as described above. The uninhibited enzyme digested approximately 50% of the substrate given i.e. within the linear part of the assays. One unit of inhibitory activity was defined as the amount of unactivated conditioned medium in μL that resulted in a 50% reduction in enzyme activity. Immunoreactive MMP-1 and TIMP-1 were determined according to the manufacturer's description.

To correct for variations in the number of cells per well, MMP and MMP inhibitor expression was normalized to the DNA content determined by the DABA method [34], where 10^6 cells corresponded to 12.2 ± 0.2 μg DNA [35]. Most MMP and MMP inhibitor assays were performed in at least duplicate on samples from three independent cell culture experiments.

Gelatin Zymography

SDS-substrate PAGE was done as described by Heussen and Dowdle [36] with gels (11 cm × 0.75 mm) containing gelatin (0.1% w/v) and 10% (w/v) polyacrylamide. Ten μL of cell-conditioned medium from 2×10^5 cells were mixed with 3μL of loading buffer (333 mM Tris-Hl [pH 6.8], 11% SDS, 0.03% bromphenol blue and 50% glycerol). Eight μL of this non-heated mixture was applied to the gel, which was run at 20 mA/gel at 4°C. Thereafter, the gel was washed twice in 100 mL of washing buffer (50 mM Tris-HCl [pH 7.5], 5 mM $CaCl_2$, 1μM $ZnCl_2$ and 2.5% (v/v) Triton X-100) and then incubated in 100 mL of assay buffer (50 mM Tris-HCl [pH 7.5], 5 mM $CaCl_2$, 1μM $ZnCl_2$ and 1 mM APMA) for approximately 20 hr at 37°C. Gels were stained with 0.2% Coomassie brilliant blue R-250 (30% methanol, 10% acetic acid) and destained in a solution containing 30% methanol and 10% acetic acid. Gelatinase activity was evident as cleared (unstained) regions. The area of the cleared zones was analysed with the GelBase/GelBlot™ Pro computer program from Ultra Violet Products (Cambridge, UK).

DNA Fragmentation

Both control, c^3Ado- and c^3Ari-treated normal human skin fibroblasts were incorporated with (^{3}H)-thymidin and tested for DNA fragmentation using the JAM DNA fragmentation test [37].

Statistical Analysis

All assays were performed in at least duplicate with the data expressed as mean (±) standard error, using the Student *t*-test.

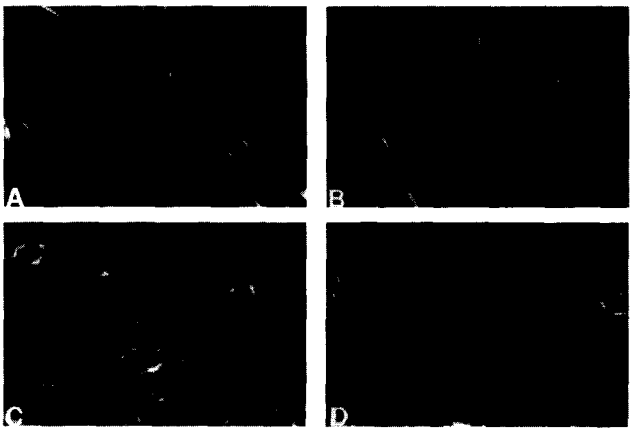

FIG. 1. Morphology of c^3Ado-treated and untreated human skin fibroblasts. 200 times magnification by light microscopy. A: Control cells; B, C, and D: 10^{-5}, 10^{-4} and 10^{-3} M c^3Ado, respectively.

RESULTS

Cell Morphology and Viability

Compared to control cells (Fig. 1A), treatment with 10^{-5} M c^3Ado induced no difference in cell morphology (Fig. 1B). Treatment with 10^{-4} M showed some uprounded cells with smaller volumes than control cells as well as some loosened cells (Fig. 1C). Treatment with 10^{-3} M c^3Ado resulted in increased amounts of loosened cells in addition to remarkable changes in cell morphology (Fig. 1D), with rounding up of cells and reduction of cell volume. Whereas some of the cells showed an extreme reduction of cell volume, most showed only minor changes. These cell changes were already observable after 6 hr treatment and remained constant up to at least 74 hr. To determine whether the observed cell changes were due to cell death or not, both treated and control cells were trypsinized, collected, washed three times in Hanks' Balanced Salt Solution (HBSS) and resuspended in serum containing DMEM. In contrast to the control cells, those treated with 10^{-3} M c^3Ado did not adhere to the culture flask, and the nonadherent cells did not divide. The viability of the c^3Ado-treated and untreated cells was also tested with the trypan blue exclusion test. Three percent of the control cells were stained, indicating that 97% of the cells were viable. Approximately 95% of the c^3Ado-treated cells incorporated the dye to varying degrees, indicating that the majority of the cells did not survive this treatment due to a damaged cell membrane. However, when the dye was added directly to the culture flask without previous trypsination, there was no difference in dye uptake by treated and untreated cells. This indicated that the damaged cell membrane in the c^3Ado-treated cells was induced by the trypsination process.

Treatment of cells with 10^{-5} to 10^{-3} M c^3Ari did not cause detectable morphological changes compared to controls. In accordance with these observations, the viability of drug-treated cells was approximately the same as that of untreated cells (data not shown).

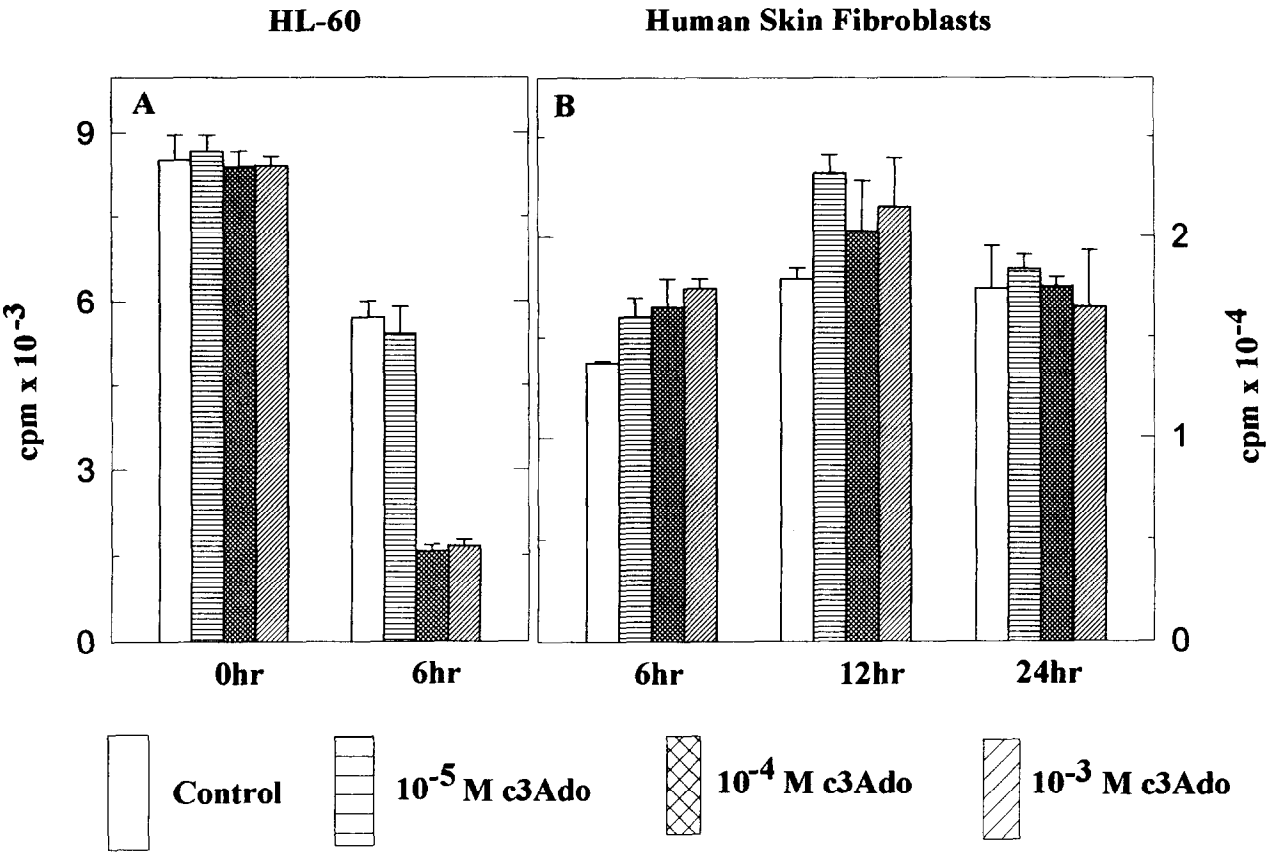

FIG. 2. Effect of c^3Ado on DNA fragmentation in HL60 cells and fibroblasts. Cells were first allowed to incorporate ^{3}H-thymidin and thereafter incubated in serum-free medium containing 0 to 10^{-3} M c^3Ado. Incubation times in hours are indicated under the graphs. DNA fragmentation was measured by the JAM DNA fragmentation test [37] and presented as mean ± S.E. (n = 6). HL-60 cells were used as positive controls. A: HL-60 cells; B: Human skin fibroblasts.

Effect of c^3Ado and c^3Ari on DNA Fragmentation

c^3Ado, but not c^3Ari, induces DNA fragmentation in HL-60 cells [24]. We investigated whether these compounds induce DNA fragmentation in normal human skin fibroblasts using c^3Ado treatment of HL-60 cells as a positive control (Fig. 2A). As shown in Fig. 2B, treatment with 10^{-5} to 10^{-3} M c^3Ado did not induce DNA fragmentation in normal human skin fibroblasts, and neither did 10^{-5} to 10^{-3} M c^3Ari (data not shown).

Collagenase, Gelatinase and Stromelysin Expression

Normal human skin fibroblasts were incubated from 6 to 74 hr in serum-free Dulbecco medium (controls) or in the same medium containing varying concentrations of c^3Ado or c^3Ari. There was no detectable collagenase activity in the culture medium from cells incubated from 6 to 11 hr. Figure 3A shows that the longer the fibroblasts were incubated in serum-free medium with or without c^3Ado, the greater the collagenase activity. After 24 hr incubation, cells treated with 10^{-3} M c^3Ado showed a significant increase in activity compared to untreated cells. The difference in activity between treated and untreated cells increased with increasing incubation time in serum-free medium. It is also noticeable that with cells stimulated for at least 50 hr, the largest increase in activity was in the 10^{-4} M c^3Ado medium. Other experiments included 10^{-5} M c^3Ado, a concentration that did not significantly alter the cell expression of collagenase activity compared to controls (data not shown). No preactivation of the collagenase enzyme could be detected in any of the cultures, and hence the cells produced only the proenzyme (data not shown).

To determine whether the increased collagenase activity in the medium from c^3Ado-treated cells was due to an increased synthesis of the collagenase protein, immunoreactive collagenase was measured in the 50 and 74 hr serum-free cell-conditioned culture medium (Fig. 3B). After 50 hr incubation in serum-free medium, there was no significant difference in immunoreactive collagenase between untreated cells and those treated with 10^{-3} M c^3Ado. A small but significant increase was obtained at 10^{-4} M c^3Ado. However, cells treated with 10^{-4} and 10^{-3} M c^3Ado for 74 hr showed a large and significant increase in immunoreactive collagenase compared to controls (Fig. 3B).

As shown in Figure 4, there was also a time dependent increase in gelatinase activity in both control and c^3Ado-treated fibroblasts. However, after 11 hr the increase in gelatinase activity was significantly lower in the drug-

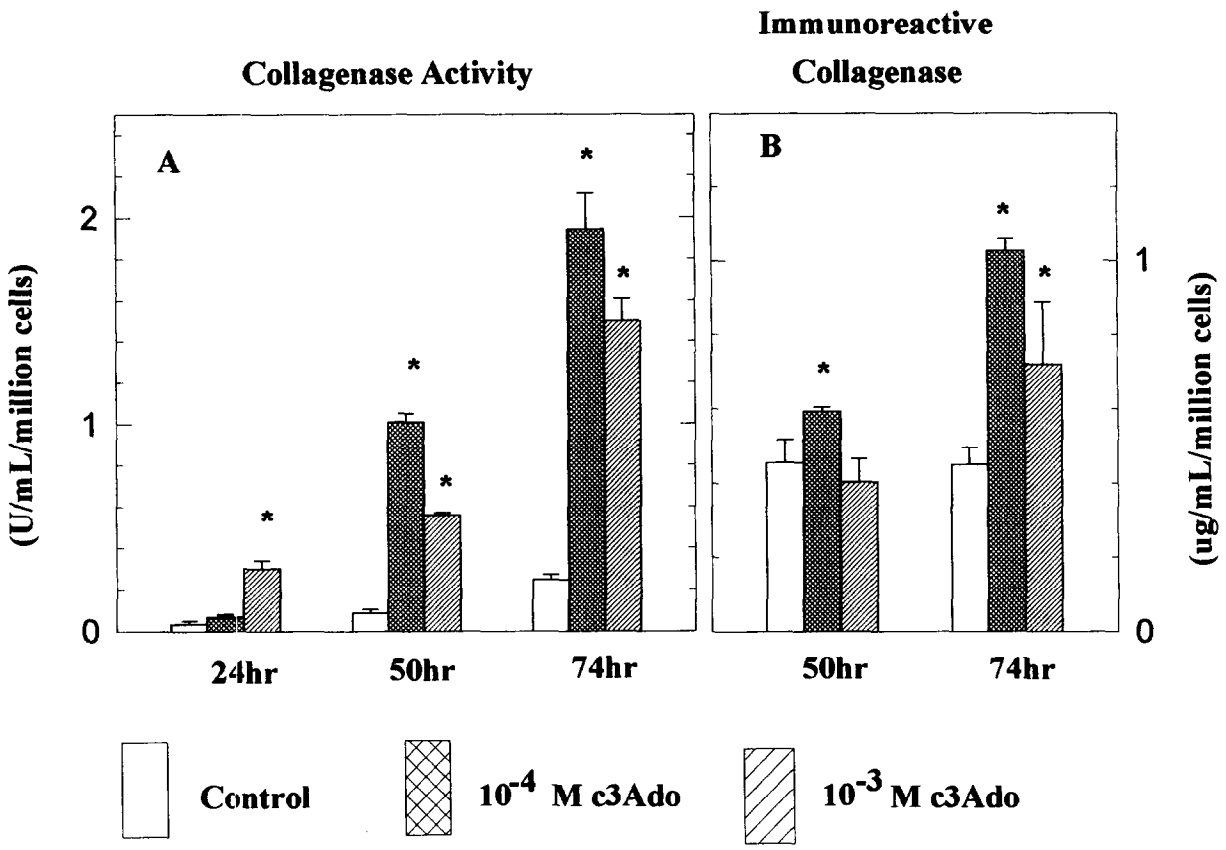

FIG. 3. Effect of c^3Ado on collagenase activity (A) and immunoreactive collagenase (B) in human skin fibroblasts. Cells were incubated in serum-free medium with increasing c^3Ado concentrations at indicated time intervals. A: Enzymes were activated with trypsin (15 min at 37°C) and the activity determined by measuring the cell-conditioned medium's ability to degrade reconstituted [^{14}C]-collagen fibrils. One U of collagenase activity is the amount of enzyme that degrades 1 μg of collagen per minute. B: Immunoreactive collagenase was mesaured by an ELISA as described by the manufacturer (Amersham). Results are presented as mean ± S.E., with n = 4 (A) and n = 8 (B); *$p < 0.05$.

treated cells compared to the controls. It is noticeable that the difference in activity between treated and untreated cells was accentuated with increasing incubation time. These changes in activity can at least in part be attributed to changes in the expression of the 72kDa gelatinase (MMP-2), as shown in the representative gelatin zymogram (Fig. 5) for cells incubated for 48 hr. Both gelatin zymography and activity methods showed that fibroblasts produced only the proenzyme.

Culture medium from c^3Ado-treated and untreated cells was also tested for stromelysin activity. There was no detectable activity in medium conditioned from 6 to 24 hr. c^3Ado induced a significant increase in stromelysin activity (Fig. 6). That the increased activity of the 10^{-3} M c^3Ado medium (50 hr) was real was verified in other experiments (48 hr), which also gave a 3-fold increase in activity with $p < 0.05$ (n = 6). These experiments also showed that the stromelysin activity in the 10^{-5} M c^3Ado medium did not significantly differ from the controls (data not shown). In the culture medium from cells incubated for 74 hr, only 10^{-4} M c^3Ado produced an increased stromelysin activity (Fig. 6). No preactivation of the stromelysin enzyme could be detected in any of the cultures, and hence the cells produced only the proenzyme (data not shown).

Culture medium from c^3Ari-treated (10^{-5} to 10^{-3} M) and untreated cells was also tested for collagenase, gelatinase and stromelysin activity. There was no significant difference between c^3Ari-treated and untreated cells with respect to these three enzyme activities (data not shown).

Collagenase and Gelatinase Inhibitor Expression

The expression of immunoreactive TIMP-1 was determined in conditioned medium from normal human skin fibroblasts treated with or without c^3Ado for either 50 or 74 hr. As shown in Fig. 7A, treatment with c^3Ado resulted in a significant decrease in TIMP-1 expression.

Investigations of collagenase and gelatinase inhibitory activity were performed after 48 hr incubation with and without 10^{-5} to 10^{-3} M c^3Ado in the serum-free medium. As shown in Fig. 7B, a significant decrease in collagenase inhibitory activity was seen in cells treated with 10^{-3} M c^3Ado compared to controls. The amount of collagenase inhibitory activity correlates well with the immunoreactive TIMP-1 in Fig. 7A.

In contrast to this, human skin fibroblasts treated with c^3Ado produced an increased amount of gelatinase inhibitor (Fig. 7C). The lowest concentration of c^3Ado (10^{-5} M)

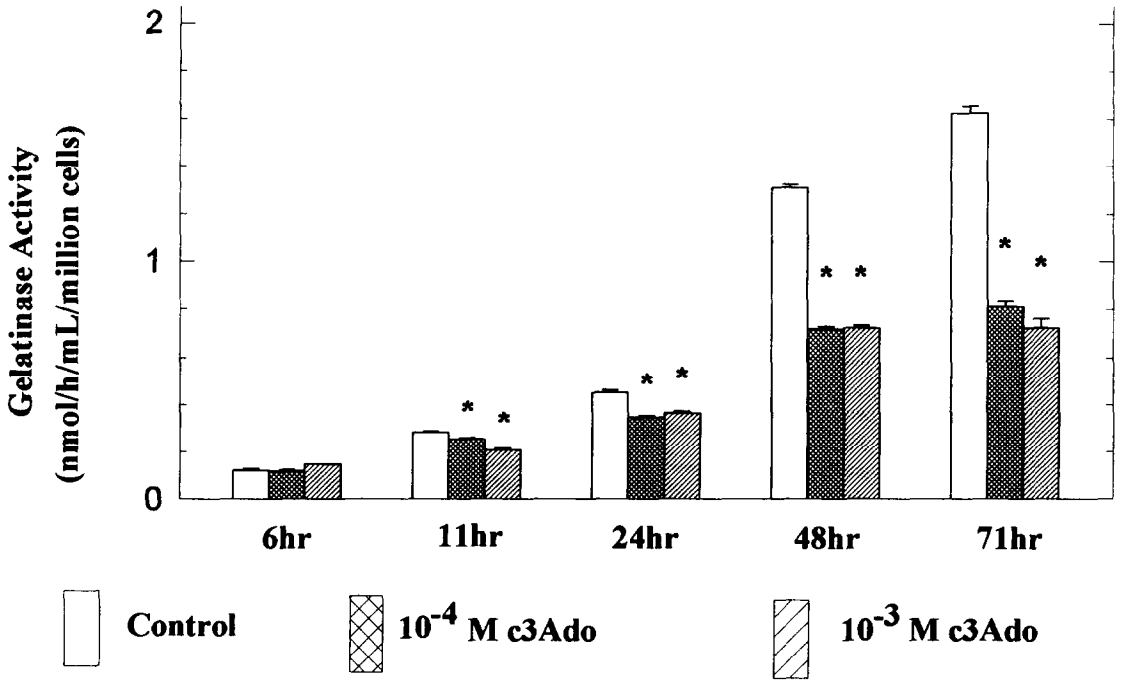

FIG. 4. Effect of c^3Ado on gelatinase activity in human skin fibroblasts. Cells were incubated in serum-free medium with different c^3Ado concentrations at the indicated time intervals. Enzymes in the conditioned mediums were activated with trypsin and aliquots were withdrawn at different time intervals (0–3 hr at 37°C) as described previously [9, 33]. The gelatinase activity was determined by measuring the cell-conditioned medium's ability to degrade heat denaturated [^{3}H]-calf skin collagen (gelatin). Results are presented as mean ± S.E, with n = 6; *p < 0.05.

showed the highest increase in inhibitory activity (approximately 70%). However, at the two highest concentrations of c^3Ado there was a small but significant increase in inhibitory activity.

No change in either collagenase or gelatinase inhibitory activity was observed in cells treated with 10^{-5} to 10^{-3} M c^3Ari compared to controls (data not shown).

DISCUSSION

It has previously been shown that c^3Ado in contrast to c^3Ari is toxic to HL-60 cells in concentrations above 50 μM, and that the mode of cell death is apoptosis [19–21, 29]. The morphological appearance of apoptosis is characterized by nuclear condensation, DNA fragmentation, blebbing of the cell surface and disintegration of the cell into apoptotic bodies [29, 38]. This is distinctly different from necrosis, which is characterized by cell enlargement and lysis. As shown in the present work, concentrations up to 10^{-5} M of c^3Ado and 10^{-3} M of c^3Ari were not toxic and did not induce morphological changes in human dermal fibroblasts. However, c^3Ado concentrations of 10^{-4} M or higher seemed to be toxic and did induce large morphological changes in the fibroblasts. The morphological changes might include changes in the microfilaments, as this adenosine analogue induced disorganisation of the microfilaments in mouse macrophages [30]. The alteration of these filaments in the macrophage cell line was a reversible process, while the morphological changes in the skin fibroblasts were associated with the induction of an apparently irreversible change to a non-proliferating phenotype, at least after trypsin treatment. The trypan blue exclusion of the c^3Ado-treated fibroblasts (i.e. prior to trypsin treat-

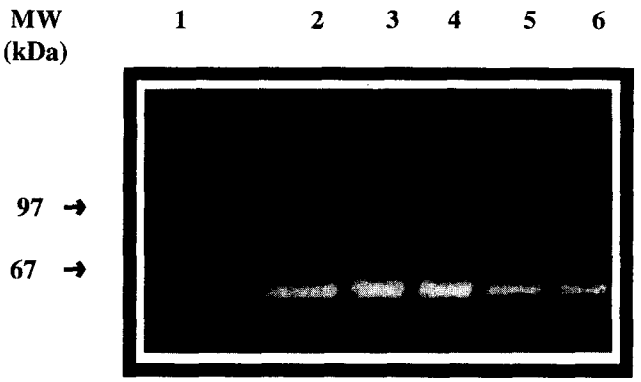

FIG. 5. Gelatin zymography of conditioned serum-free medium from fibroblasts treated with c^3Ado concentrations from 0 to 10^{-3} M for 48 hr. The conditioned medium was treated and applied to electrophoresis as described in the Methods section. MW standard in Lane 1; an untreated control fibroblast strain in Lane 2; the untreated control fibroblasts in Lane 3; cells were treated with the following concentrations of c^3Ado: 10^{-5} M in Lane 4; 10^{-4} M in Lane 5; and 10^{-3} M in Lane 6. To estimate the amount of gelatinase activity and hence the amount of gelatinase protein in the 66kDa zone, i.e. MMP-2, the area of each cleared zone was determined as described in Methods. The relative amount in percent compared to the control in Lane 3 was as follows: 98% in Lane 4; 78% in Lane 5; and 64% in Lane 6.

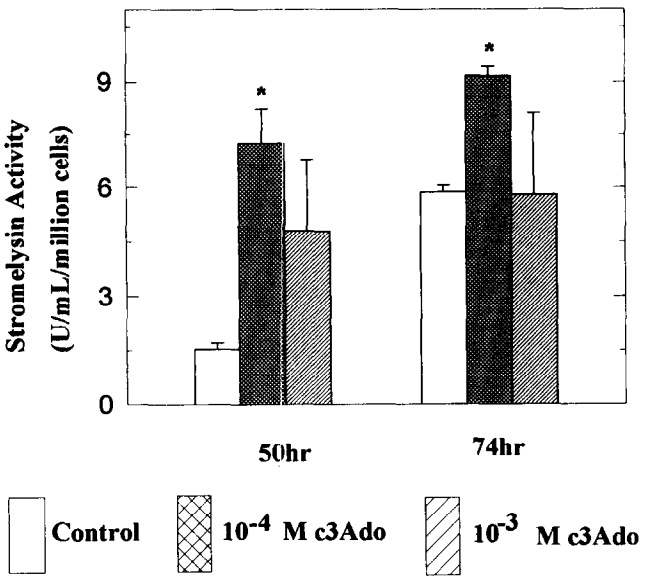

FIG. 6. Effect of c^3Ado on stromelysin activity in human skin fibroblasts. Cells were incubated in serum-free medium with different c^3Ado concentrations at the indicated time intervals. Enzymes were activated with trypsin (30 min at 37°C) as described previously [9, 33]. The stromelysin activity was determined by measuring the cell-conditioned medium's ability to degrade azocasein, and IU of enzyme activity is the amount of enzyme that causes a change of 0.001 A_{440nm} per minute. Results are presented as mean ± S.D. with n = 2; *$p < 0.05$.

ment) indicated that the morphological changes and lack of viability were not associated with membrane damage and necrosis. Other signs of necrosis such as increase in cytoplasma and cell volume followed by lysis were not observed. On the contrary, cells shrank as a possible sign of apoptosis, whereas other apoptotic characteristics such as DNA fragmentation and cell blebbing were missing. As previous studies have shown that DNA fragmentation is not a prerequisite for apoptotic cell death [39, 40], the lack of viability of the drug-treated fibroblasts may be due to either apoptosis, induction of an apoptotic-like process, or other deterioration processes. Another possible explanation is that c^3Ado causes an alteration that predisposes the cells to irreversible damage upon subsequent treatment with trypsin.

The two adenosine analogues studied in the present work are known to be indirect inhibitors of methylation, thymocyte proliferation, prostaglandin release and RNA synthesis [22, 27, 41, 42]. At non-lethal concentrations, c^3Ado in contrast to c^3Ari did reduce the expression of c-myc, v-fos, actin and histon H2B mRNA [42]. The two adenosine analogues also induced an increased expression of α1(IV) collagen mRNA [43]. The present work shows that c^3Ari and c^3Ado at non-toxic concentrations had no effect on MMP and MMP inhibitor production, with the exception of the increased expression of gelatinase inhibitors at 10^{-5} M c^3Ado. However, c^3Ado concentrations that induced marked morphological changes and non-viable fibroblasts also induced an altered expression of MMPs and their inhibitors. It is noticeable that these changes in MMP and MMP inhibitor expression were accentuated with increased incubation time in serum-free medium (Figs. 3, 4, 6, 7). These results show that although the c^3Ado-treated cells appeared to be non-viable and not able to attach to the plastic culture plate and divide in reculturing experiments, they did retain their ability to differentially express proteins. The mechanism behind the c^3Ado-induced increase in collagenase activity varied with the concentration of the adenosine analogue and the incubation time in serum-free medium. In cells incubated with 10^{-4} M c^3Ado for 50 hr, the increase in collagenase activity could be attributed to three different events: an increased synthesis of the collagenase protein, a decreased synthesis of the collagenase inhibitor TIMP-1, and increased stromelysin production (Figs. 3, 6, 7). However, in cells treated with 10^{-3} M c^3Ado for 50 hr, the activity increase was only due to two events: a reduced TIMP-1 level and increased stromelysin activity (Figs. 3, 6, 7). The altered collagenase activity after 72 hr treatment with 10^{-4} M of the adenosine analogue was also due to two events: an increased synthesis of both the collagenase protein and the superactivator stromelysin (Figs. 3, 6, 7). A fourth constellation appeared in cells treated with 10^{-3} M c^3Ado for 72 hr, i.e., an elevated level of the collagenase protein and a decreased level of TIMP-1. It is noticeable that the trypsin- or APMA-activated collagenase activity could be up to 12-fold enhanced in the presence of stromelysin, a phenomenon known as "superactivation," i.e. stromelysin acts in synergy with other activators [44]. That stromelysin alone can be a regulator of a cytokine-induced change in collagenase activity was shown by Unemori et al. [45]. Interferon-γ downregulated interleukin-1β-induced collagenase activity by reducing stromelysin expression, while the level of the collagenase and the TIMP-1 protein was unaltered [45]. Both Unemori's and our work show some of the complex mechanisms behind a cytokine- or drug-induced regulation of collagenase activity.

Figure 7 shows that c^3Ado treatment also resulted in the increase of an inhibitor that seems to affect gelatinase activity, but not collagenase activity. The reduction in gelatinase activity can at least in part be attributed to an increased production of a gelatinase inhibitor and a decreased production of the gelatinase (MMP-2) protein (Fig. 5). As the two adenosine analogues acted differently on the dermal fibroblasts, it seems unlikely that the effects induced by c^3Ado can be attributed to a general inhibition of methylation reactions. The altered gene expression is most likely caused by other mechanisms, including the formation of c^3AdoHcy which may have targets that differ from AdoHcy. Another possibility is the disorganisation of the cellular microfilaments, analogous to the mechanism by which colchicine and other microtubule disrupting agents induce an increased expression of collagenase in human dermal fibroblasts [31, 32]. Independent of the detailed mechanism behind the c^3Ado-induced modulation of MMP and MMP inhibitor expression, it is noticeable that

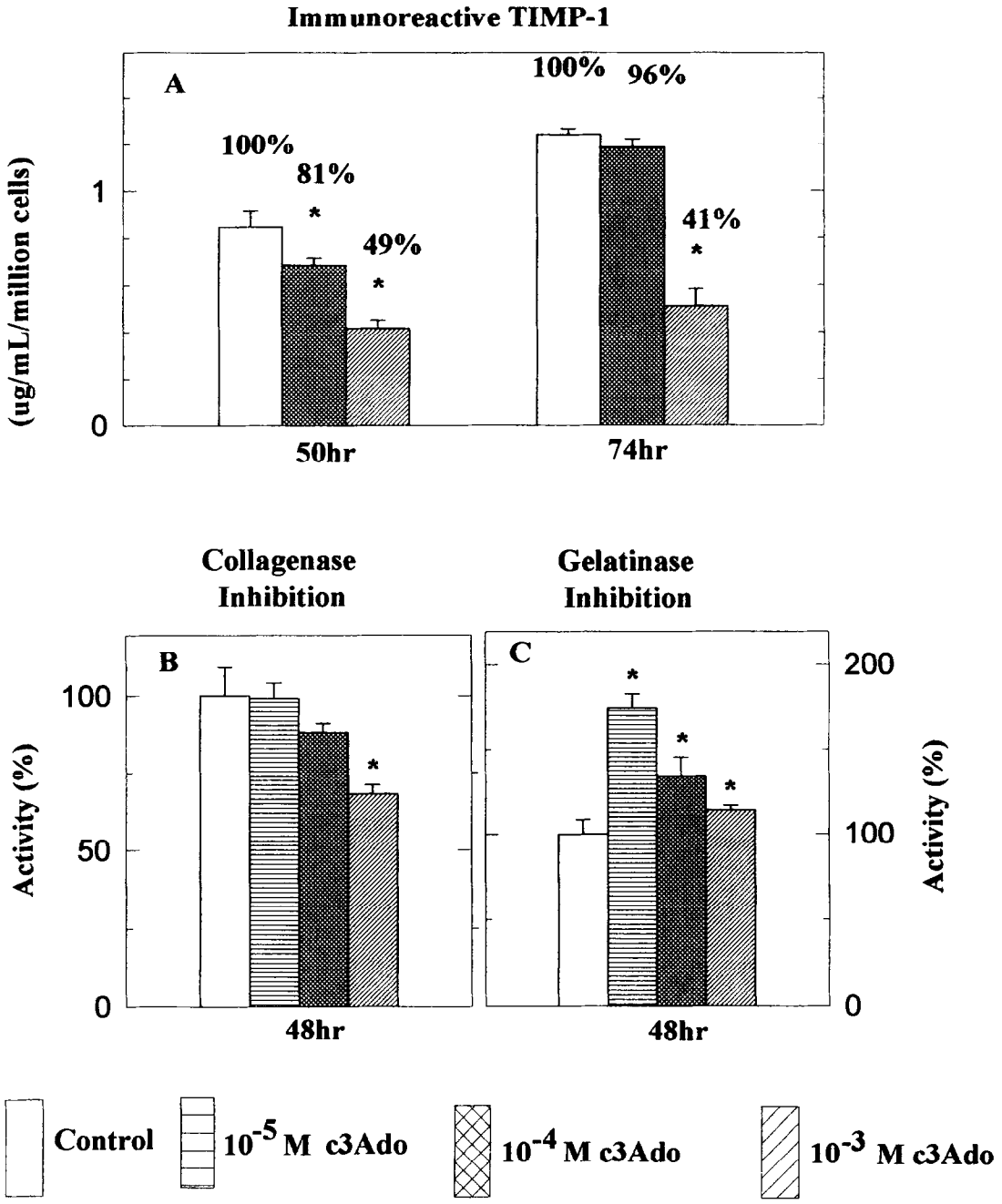

FIG. 7. Immunoreactive TIMP-1, collagenase and gelatinase inhibitory activity in cultured medium from human skin fibroblasts untreated or treated with c^3Ado. The incubation time of the cells in serum-free medium is indicated under the graphs. A: Imunoreactive TIMP-1 was measured in the different conditioned mediums by an ELISA as described by the manufacturer (Amersham). B: The amount of collagenase inhibitory activity in the culture medium was determined as follows: A large batch of conditioned medium with known high collagenase activity was activated with trypsin (15 min at 37°C). Twenty-five μL of the activated medium were mixed with increasing amounts of un-activated medium, giving a total volume of 100 μL [9, 33]. Collagenase activity was measured as described under Methods. C: The amount of gelatinase inhibitory activity in the culture medium was determined as follows: A large batch of conditioned medium was activated with trypsin (120 min at 37°C). Twenty-five μL of the activated medium were mixed with increasing amounts of un-activated medium, giving a total volume of 200 μL [9, 33]. Gelatinase activity was measured as described under Methods. The results are presented as mean ± S.E. with n = 8 (A), n = 4 (B) and n = 6 (C); *$p < 0.05$.

this compound can modulate both the expression of structural extracellular matrix proteins (type IV collagen) [43] and the activity of the proteolytic enzymes that regulate their turnover.

The two adenosine analogues used are both potent anti-viral and cytotoxic agents, while c^3Ado also has anti-inflammatory properties [25–28, 31]. They are also potential anti-cancer drugs that differ in cytotoxicity in

both animal models and in cancer cell lines [19–21, 23, 24, 26]. The changes induced in the normal human dermal fibroblasts by c^3Ado may at least in part explain some of its *in vivo* toxicity as well as some of the necessary metabolic changes a dermal cell line may undergo during a drug-induced or controlled/programmed cell death *in vivo*. As collagenase initiates and controls the degradation of interstitial collagen, induction of collagenase activity in the poisoned and apparently dying cells may be due to the need for the entrance of phagocytic cells to the decaying area. Gelatinase degrades not only denatured interstitial collagen (gelatin), but also the intact triple-helical region of collagen IV, the main component of basement membranes [1–3]. A decreased expression of gelatinase activity may prevent an uncontrolled degradation of other connective tissue and basement membrane components. The gelatin produced through the initital attack of collagenase on interstitial collagen would be digested to shorter fragments by enzymes that include gelatinase as well as slowly formed low MW forms of autoactivated/degraded collagenase that have lost their ability to degrade intact interstitial collagen [46]. *In vivo* experiments on mice showed that c^3Ado was more toxic than c^3Ari [23, 24, 26], which might to some extent be explained by their different effect on dermal cells as described in the present work.

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