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Effect of a Monocyte Cell Factor (MCF) on Collagen Production in Cultured Articular Chondrocytes : Role of Prostaglandin E_2

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Summary : A monocyte cell factor (MCF) inhibited the incorporation of (^{3}H)-proline into collagen of rabbit articular chondrocytes in culture, without significant effect on non-collagen protein. In addition, MCF produced a new compartmental repartition of collagen between cell layer and medium. No MCF-induced shift was observed in the relative proportion of collagens synthesized, type II remaining the major collagenous product. The inhibitory effect of MCF was not completely suppressed when prostaglandin synthesis was blocked by indomethacin. Addition of PGE_2 at 12.5-25 µg/ml to the cultures resulted in a decrease of total collagen. Lower concentrations (0.42-0.85 µg/ml) did not affect the total synthesis of collagen but changed its distribution between cells and medium in the same way as MCF. These results suggest that the MCF-stimulated release of PGE_2 may be partially involved in the inhibitory effect observed on collagen synthesis.

Introduction : Monocyte and macrophage-derived factors have been shown to induce collagenase and neutral metallo-proteinases secretion by synovial cells in culture (1-2). These results may help to understand the mechanisms involved in the destruction of articular cartilage occuring in rheumatoid arthritis. In addition to enzymatic catabolism, impaired synthesis of connective tissue macromolecules might contribute to the cartilage breakdown. In this respect, we have shown that a monocyte cell factor previously isolated by Dayer et al. (3) could depress the collagen secretion and stimulate that of glycosaminoglycans in cultured synovial cells (4).

It has also been evidenced that the cartilage itself may take a part in the destructive process (5). The articular chondrocytes can produce collagenase and neutral proteinases under the influence of a macrophage factor (6).

Abbreviations : MCF: Monocyte cell factor; DMEM: Dulbecco's modified Eagle medium; FCS: fetal calf serum; PBS: phosphate buffer solution; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA: trichoracetic acid.

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Furthermore, recent data reports that conditioned medium from mononuclear cells decreases collagen synthesis in suspension cultures of chondrocytes (7).

Here, we report the effect of a partially purified monocyte factor on the collagen production in monolayer cultures of rabbit articular chondrocytes. Since monocyte or macrophage factors stimulate the prostaglandin E_2 release in synovial cells (1) as well as in chondrocytes (8-9), that prostaglandin accumulation might account for the MCF-induced collagen decrease (10-11). So, it was of interest to investigate also the effect of exogenous PGE_2 on collagen production and the action of the MCF when cells were treated with indomethacin to inhibit the prostaglandin synthesis.

Methods :

Chondrocyte culture - Articular cartilage slices were taken from the shoulders and the knees of 1-2 months old rabbits. Chondrocytes were enzymatically released (12) and cultured in DMEM supplemented with 10 % FCS, 100 I.U./ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml Fungizone. The cells were grown at 37°C in a 5 % CO_2 environment and experiments were performed on primary confluent cultures.

Preparation of monocyte cell factor (MCF) - Human blood mononuclear cells were isolated and cultured for 3 days according to Dayer et al. (1) in DMEM + 10 % FCS, antibiotics and 5 µg/ml phytohaemagglutinin (Serva). The MCF was partially purified from the conditioned medium by Ultrogel AcA 54 (LKB) filtration (3) and kept frozen at -20°C until use.

Collagen labelling, assay and purification - Exposure of chondrocytes to MCF was generally performed on a 3 day period. (^{3}H)-labelled collagen was produced by incubating cultures for the last 48 h in 3 cm Petri dishes with 1.5 ml DMEM with 10 % FCS containing 5 µCi (^{3}H)-proline (CEA France, 20-30 Ci/mM), 100 µg/ml ascorbic acid and 100 µg/ml B-aminopropionitrile. At the end of the incubation, the media and three rinses (1 ml PBS) of the cell layers were pooled and added to an equal volume of cold 10 % TCA, 2 mM proline. The cell layers were scrped with a rubber policeman, the dishes washed twice with 1 ml PBS and the pooled extracts treated with TCA as the culture medium after addition of the same volume of FCS to aid pellet formation. The precipitates were collected by centrifugation, washed three times with cold 5 % TCA, 1 mM proline and the (^{3}H)-collagen and non-collagen labelled protein were determined with purified bacterial collagenase (13). The radioactivity of all samples was determined using ACS solution (Amersham). All counts were quench corrected.

Collagen phenotypes were determined by labelling cultures in 75 cm^2 Falcon flasks in the same medium supplemented with 50 µCi/ml (^{3}H)-proline. Collagen was isolated from the whole culture (cells + medium) by pepsin treatment followed by neutral and acid salt-precipitation (12). Collagen chains were then fractionated by SDS-PAGE on 6.5 % slab gels (14). Fluorography of the gels was performed with En^{3}Hance (Amersham) (15) and the radioautographs were scanned using a densitometer (Helena).

Addition of drugs - Indomethacin (Sigma) was dissolved in a small volume of ethanol and diluted with PBS to give a 10^{-2} M solution which was added to the culture medium.

Prostaglandin E_2 (Sigma) was dissolved in ethanol (2 mg/ml) and added to the medium with the final concentration of ethanol generally kept below 0.2 %. When the prostaglandin concentration was increased to 25 µg/ml, controls were made with the same ethanol concentration (1.25 %).

Results : Significant inhibition of collagen synthesis (Fig. 1A) occured when chondrocytes were incubated for three days with MCF (1:10). As for

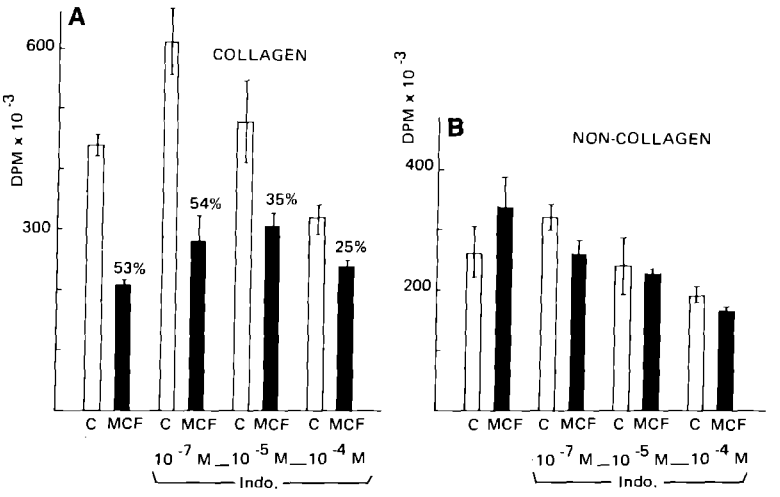

Figure 1. Effect of MCF and Indomethacin on the incorporation of (^{3}H)-proline into macromolecules. Confluent chondrocytes were incubated for 3 days in the presence or absence of MCF (1:10) and the association MCF-Indomethacin. Labelling was performed with 5 µCi (^{3}H)-proline during the last 48 h of the incubation period. Collagen (panel A) and non-collagen protein (panel B) were assayed as described in Methods. Results are presented as dpm/dish. The extentof inhibition is indicated on the top of the bars, as % of each respective control. Each value represents the mean ± SE of at least three determinations. C: controls.

synovial cells (4) this effect was dose-dependent (data not shown). In contrast, non-collagen protein remained relatively unaltered (Fig. 1B), so that the percent of collagen to total protein declined. The effects produced by indomethacin alone and the association of the latter with MCF are depicted in Fig. 1. A decrease of the collagen production was observed for 10^{-4} M indomethacin, a dose generally used to inhibit the prostaglandin synthesis in various cellular systems, including articular chondrocytes (16-17). Two lower concentrations of indomethacin (10^{-5} - 10^{-7} M), probably unsufficient to completely suppress the cyclooxygenase activity, were used. No effect was obtained with 10^{-5} M indomethacin but a 10^{-7} M solution stimulated the collagen synthesis by 38 %.

In presence of the drug, MCF still depressed the (^{3}H)-collagen amount produced, but to a lesser extent in 10^{-4} M indomethacin-treated cells. On the other hand, no great modification was observed in the non-collagen protein level when the chondrocytes were exposed to the drug or to the association drug-MCF (Fig 1B).

As shown in Fig. 2, MCF influenced the collagen distribution between culture medium and cell layer. Whereas the culture medium radioactivity remained relatively constant, the (^{3}H)-proline incorporation was significantly reduced in the cell layer. Addition of indomethacin to the cultures did not alter this distribution, nor did the association indomethacin + MCF.

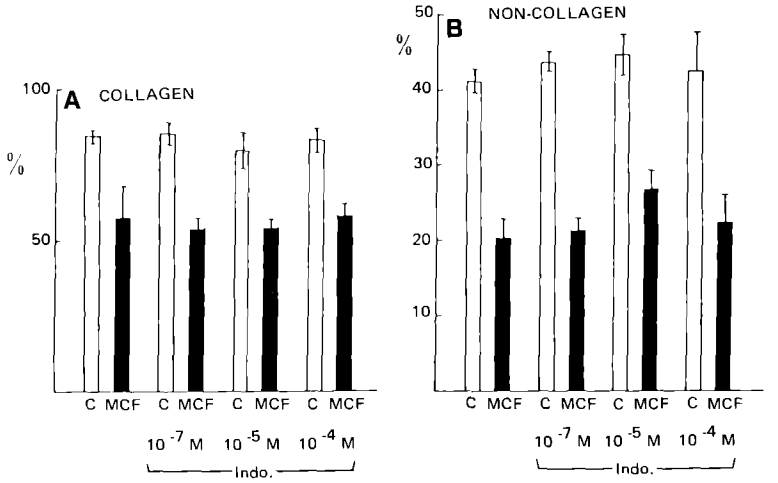

Figure 2. Cell layer collagen and non-collagen radioactivity expressed as percent of the total amount produced. Experimental conditions as in Fig. 1.

The non-collagen protein repartition varied in the same manner as that of collagen (FiG. 2B).

SDS-PAGE of the labelled collagen chains showed that the bulk of radioactivity was recovered in the position of control α_1 chains with few detectable α_2 chains (Fig. 3). Thus, the collagen secreted was essentially type II collagen, suggesting that these chondrocytes were fully differentiated. However, in addition to type II collagen, other "minor" collagen chains were detected. Two bands with lesser mobility than α_1 chains were observed which probably correspond to two of the 1α, 2α or 3α collagen chains reported previously in cartilage (18). Furthermore, the electrophoregrams showed a light band in a position consistent with that of the 59 K collagen recently discovered in cultures of chick chondrocytes (19). The material to be seen on the top of the gel disappeared after reduction of the samples. If it was type III collagen it would be recovered in position of α_1 band. However, this latter is so faint that any small variation could not be detected. Thus, we cannot exclude a possible presence of a slight amount of type III collagen.

From the fluorogram scanning, no significant difference was observed between samples from controls and MCF-treated cells (Table 1). However, the light "minor" collagen band preceding α_1 chains disappeared in patterns from cells exposed to MCF. Here too, the radioactivity of this material was so small ($\pm$ 1 % of the total amount) that more precise analysis is required to get a clear-cut conclusion. Within the limits of the methods used, there was an inhibition of the overall net collagen synthesis without change in the type distribution.

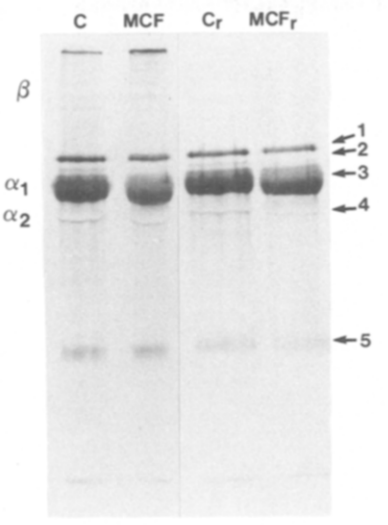

Figure 3. SDS-polyacrylamide gel (6.5 %) electrophoresis of total labelled collagen (cells + medium), without and with B-mercaptoethanol reduction. Chondrocytes were incubated for 3 days in presence or absence of MCF (1:10) and 50 μCi/ml of (^{3}H)-proline. Collagen was extracted as described in Methods. Aliquots of same total dpm values were compared. Positions of α_1, α_2, and β chains of type I collagen are indicated. The densities of the bands numbered 1, 2, 3, 4 and 5 are given in the Tab. I as percent of total band scanning intensity.

To test the hypothesis that the MCF-induced inhibition of collagen synthesis might occur as a response to the stimulated PGE_2 release, the effect of various doses of PGE_2 on the collagen labelling was studied in the same cultures. Confluent cells were routinely used to avoid the inhibitory effect of PGE_2 on cell proliferation. Since prostaglandin action on cells generally occured within a few hours, experiments were carried out on a 24 h incubation period. Low concentrations of PGE_2 (0.42-6.75 μg/ml) did not significantly affect the total amount of collagen secreted. Inhibition was observed only for 25 μg/ml PGE_2 (Fig. 4A).

All PGE_2 concentrations used were ineffective on non-collagen protein synthesis. The slight depression observed for 6.75-25 μg/ml was not significant when compared to the ethanol-containing controls (Fig. 4B).

TABLE 1 : Ratios of collagen chains calculated from densitometer tracings

	Electrophoresis bands				
	1 %	2 %	3 (α_1) %	4 (α_2) %	5 %
Control	14.42 ± 1.22	0.98 ± 0.15	80.92 ± 3.11	1.15 ± 0.21	2.53 ± 0.68
MCF	13.16 ± 1.93	0.20 ± 0.02	82.83 ± 2.72	0.91 ± 0.15	2.90 ± 0.81

Results are expressed in percent of total bands density. Values are mean of 3 replicates ± SE.

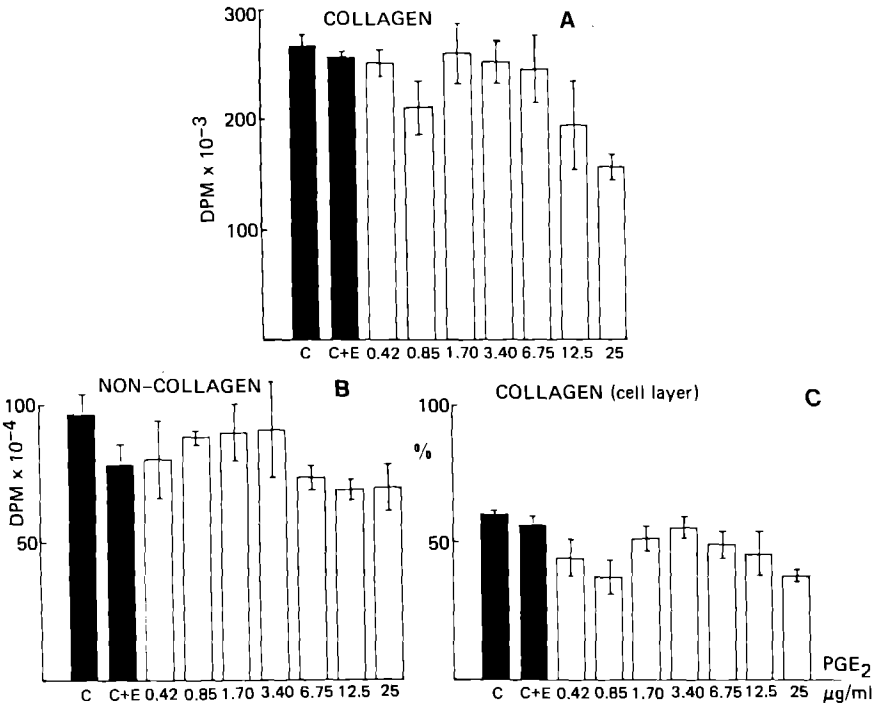

Figure 4. Effect of PGE_2 on the incorporation of (^3H)-proline into macromolecules. Confluent chondrocytes were incubated for 24 h with various concentrations of PGE_2 and 5 μCi (^3H)-proline. Controls containing 1.25 % ethanol, concentration corresponding to that of cells incubated with the highest dose of PGE_2 (25 μg/ml) were introduced (C + E). C: Controls without ethanol. Panels A and B: results are presented as dpm/dish. Each value represents the mean ± SE of at least three determinations. Panel C: Cell layer collagen as percent of the total secreted collagen.

As shown in Fig. 4C, the distribution of labelled collagen between medium and cell layer was not altered by addition of 1.25 % ethanol. On the other hand, for the low concentrations of PGE_2 (0.42-0.85 μg/ml), the medium fraction was increased above the control levels although the total amount collagen (cells + medium) remained unchanged, so that the relative proportion of cell layers declined. Then, a distribution approximately equal between cell layer and medium was obtained for higher doses of PGE_2, except for 25 μg/ml where medium percent increased again. In contrast, no significant change was detected in non-collagen protein compartmental distribution whatever the concentration of PGE_2.

Discussion : In previous studies, we have shown that mitogen-stimulated mononuclear cell supernates decreased human synoviocyte collagen synthesis in a dose-dependent manner (4). The results presented here clearly demonstrate that rabbit articular chondrocytes respond in the same way to the semi-purified monocyte cell factor already known to stimulate collagenase and PGE_2 secretion in both synovial cells (1) and chondrocytes (8). Our observations are comparable to those obtained by Herman et al. (7) using

chondrocyte suspension cultures and lymphokine-rich conditioned media. Like these authors, we have also shown that collagen is more sensitive to MCF than non-collagen protein synthesis, the latter being rather unaltered.

An important point to be emphasized is that MCF produces a new distribution of collagen and non-collagen protein between cell layer and culture medium. Interestingly, this phenomenon has also been reported recently for suspension cultures of chondrocytes (7) where there is not a true insoluble matrix between cells as in monolayer cultures. This MCF-induced modification of the pericellular environment may reflect changes in membrane properties, biophysical characteristics of macromolecules synthesized or relative proportion of these latter. Whatever the mechanism be, the question arises whether these observations have a pathophysiologic significance and could provide new insights into the destructive mechanisms of cartilage occuring in various articular diseases. Further investigation is required to clarify these points.

Articular chondrocytes in culture may undergo phenotypic variations and synthesize type I collagen in addition to type II which is specific of cartilage (12). Variation in the collagen type ratio of cartilage, with presence of type I collagen, has also been reported in association with articular diseases such as osteoarthritis (20). Such a dedifferentiation of chondrocytes may contribute to the catabolic events leading to pathological destruction of cartilage. Thus, it was of interest to investigate whether the collagen type of chondrocytes could be influenced by the MCF.

From our results, the phenotypic expression of collagen was not altered by the presence of MCF in the cultures, at least for a 3-day incubation period. In particular, no switch from type II to type I took place as it has been described for dedifferentiated chondrocytes (12). Furthermore, since comparative electrophoreses were performed with samples containing the same radioactivity and the relative proportions of the resulting bands did not change, we may deduce that MCF affects the synthesis of each collagen type. This result does not correlate with recent study from Trechsel et al. (21) who found that mononuclear cell supernate specifically inhibits type II collagen synthesis, that of type I being unchanged. It must be noted that these authors used articular chondrocytes which apparently synthsized a higher amount of type I collagen than did our cultures, perhaps because their experiments were performed on first subcultures instead of primary cultures. In our Study, if type I synthesis was unchanged by MCF-treatment, the ratio of the α_2 to α_1 bands should have been increased comparatively to controls since there is an overall inhibition. This was not the case. However, the α_2 band is so light that small variation of this material cannot be accurately determined.

If the expression of type II collagen is not altered by exposure of cells to MCF, we cannot exclude that subtle differences may affect the "minor" collagen chains ratio. Since most of these collagens contribute to the pericellular domain, such small qualitative or quantitative variations would be perhaps meaningful to explain the MCF-induced redistribution of macromolecules between cell layer and culture medium. More precise analysis is needed to elucidate this question.

The mechanism of the overall net decrease in collagen production is not clear for the moment. It could be considered as the result of both synthesis inhibition and catabolism enhancement. However, it is unlikely that extracellular proteolytic activity might contribute, at least in part, to the collagen decrease since culture medium contained 10 % fetal calf serum which can inhibit most of catabolic enzymes such as collagenase. More probably, an intracellular degradative process could be involved, as preliminary results from our laboratory have revealed that MCF enhances the intracellular degradation of newly synthesized collagen. This increase appears as a secondary desponse to a MCF-induced rise in the level of cellular cAMP (22). This view is supported by the fact that cAMP has been shown to stimulate the intracellular degradation of collagen in fibroblasts (23).

The ability of MCF to depress the amount of label incorporated into the collagen might be due to a decrease in the specific activity of the intracellular proline or prolyl-tRNA pool. This possibility will require further investigation.

PGE_2 is the major prostaglandin produced by monolayer cultures of chondrocytes (24). Since this secretion can be greatly stimulated by MCF-treatment (8), it would be conceivable that PGE_2 accumulation could be responsible for the collagen modulation observed in our experiments. This view is supported by the correlation which has been observed between the biosynthesis of prostaglandin and cyclic AMP in similar cultures of rabbit articular chondrocytes (17). However, the role of PGE_2 on the biosynthesis of collagen in chondrocytes is not clear for the moment. Some reports have shown an inhibitory effect of this prostaglandin on the incorporation of ^3H-proline in the cartilage (25-26) or more generally on the protein synthesis (27). However, in most of these studies the concentrations required to obtain a response were rather high. Our results confirmed the low sensitivity of the chondrocyte collagen synthesis to exogenous PGE_2 since a 25 µg/ml dose was needed to observe an inhibitory effect. Such a concentration is far much greater than the endogenous production of cultured articular chondrocytes (24). Therefore, the addition of exogenous PGE_2 in our experiments only partially mimics the MCF effect on collagen synthesis. However, an

interesting finding of our study was that the lowest doses of PGE_2 (0.40-0.85 μg/ml) did not affect the overall net collagen production but decreased the relative amount in the cell layer as did the MCF. This must be related to a recent study (17) showing that a stimulation of cAMP synthesis can be observed in similar chondrocyte cultures after addition of only 1 μg/ml PGE_2. Such a result would indicate that a small enhancement of cAMP level might be sufficient to affect the distribution of collagen between cells and medium whereas higher increase might be required to influence the production itself. This point need futher investigation.

Results from our laboratory have shown that the addition of PGE_2 to chondrocytes in association with 10^{-4} M indomethacin did not reveal any increased sensitivity, at least on collagen synthesis, of these cells when the endogenous PG synthesis is blocked (not shown). On the other hand, Dayer et al. (22) reported that incubation of synovial cells with MCF and indomethacin together resulted in a potentiation of cAMP response to exogenous PGE_2 greater than that observed with cells pre-incubated with indomethacin alone. This data clearly indicated that MCF increases the responsiveness of the cells to PGE_2. Such a mechanism has been also suggested for the action of MCF on human articular chondrocytes (8).

Assuming that indomethacin is acting as a cyclooxygenase inhibitor, the reduction observed here in the effect of MCF on collagen synthesis when chondrocytes were treated by 10^{-4} M indomethacin stongly suggests that PGE_2 is partially involved in the mechanism. The PGE_2 produced by MCF-treatment of the chondrocytes may stimulate the adenylate cyclase of these cells, resulting in cAMP biosynthesis which, in turn, would be responsible for the collagen inhibition (10). This view is supported by the presence in chondrocytes of a membrane-bound adenylate cyclase particularly responsive to PGE_2 (8).

However, indomethacin failed to completely suppress the MCF effect on collagen biosynthesis. Thus, we cannot eliminate the possibility that other (s) mechanism (s) not related to PGE_2 synthesis may participate to the MCF-modulation of collagen produced by chondrocytes. An other way in which MCF effect might be exerted could be the synthesis of lipoxygenase products from arachidonic acid, such as leukotrienes but this speculative interpretation remains to be clarified. In fact, indomethacin influences the collagen biosynthesis of chondrocytes by a mechanism which seems not very clear. For the moment, no simple explanation can be given to the fact that this drug produced a stimulation at a concentration of 10^{-7} M whereas it exerted an inhibition at 10^{-4} M. Furthermore, if PGE_2 is considered to have a depressing effect on collagen production, the blockade of PG synthesis by

10^{-4} M indomethacin would have resulted in a stimulation instead of an inhibition. In addition to its inhibition of the cyclooxygenase pathway, indomethacin could operate through unrelated pharmacologic mechanisms. Of special interest in this regard is the report that this drug alters PGE binding to murine hepatocytes by lowering the binding affinity of their PGE receptors (28) and that effect seems to be unrelated to cyclooxygenase inhibition.

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