

Modulation of 72-Kilodalton Type IV Collagenase (Matrix Metalloproteinase-2) by Ascorbic Acid in Cultured Human Amnion-Derived Cells¹

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ABSTRACT

Extensive research has been done to investigate the effects of nutrients on placental and fetal development. It is now evident that environmental factors such as diet may exert a profound effect on gene expression during pregnancy. A low intake of vitamin C during pregnancy has been linked to a higher risk of premature rupture of the membranes (PROM) because of its well-known role in collagen biosynthesis. Here we report a new effect of ascorbic acid acting as a modulator of the 72-kDa type IV collagenase (matrix metalloproteinase-2; MMP-2). MMP-2 expression/activity is down-regulated by vitamin C in human amnion cultured cells. The regulatory effect is exerted at the transcriptional level and is specific for MMP-2. Matrix metalloproteinases are implicated in tissue remodeling, and our results allow us to suggest a molecular mechanism that relates poor availability of vitamin C during pregnancy and the development of PROM.

INTRODUCTION

Premature rupture of chorioamniotic membranes (PROM) is an obstetric complication that is present in 2–17% of all pregnancies [1]. The highest prevalence of PROM has been linked to populations with low socioeconomic conditions [2]. As a consequence, the search for causal determinants of PROM has focused on factors associated with adverse socioeconomic status, such as urogenital infections and malnutrition and/or specific dietary deficiencies [3]. The participation of vitamin C in the development of PROM has been suggested owing to its well-described role in the biosynthesis of collagen, the main structural constituent of chorioamniotic membranes [4]. This hypothesis has been partially supported by the findings of low mechanical resistance and decreased collagen concentrations in samples of fetal membranes that ruptured prematurely [5–7].

An association between marginal deficiency of vitamin C and PROM was found in a cross-sectional study [8]. In addition, a relationship between low concentrations of leukocyte ascorbic acid at Week 28 of pregnancy and later development of PROM was reported in a recent prospective study [9]. This background, and the fact that pregnancy implies an extra demand of nutrients, suggest that marginal deficiency of vitamin C should be further studied.

A novel physiopathogenic mechanism for PROM, involving increased collagen degradation in chorioamnion

mediated by matrix metalloproteinases (MMPs), has been recently proposed [10]. MMPs, such as collagenase, stromelysin, and type IV collagenases/gelatinases, are a group of enzymes that are involved in extracellular matrix catabolism [11]. This family of enzymes plays a key role in connective tissue turnover and has been implicated in many normal and pathological processes. These enzymes are synthesized by chorioamniotic cells, and MMP-2 or 72-kDa type IV collagenase is a constitutive enzyme during pregnancy [12, 13]. It seems that collagen degradation plays an important role in PROM; therefore the main objective of this study was to assess whether ascorbic acid may exert a direct action on collagenolysis using cultured chorioamniotic membrane resident cells as a study model of extracellular matrix catabolism modulation by vitamin C.

MATERIALS AND METHODS

Cell Culture

WISH cells, a line of human amnion cells (American Type Culture Collection, Rockville, MD; ATCC CCL5) were cultured in 25-cm² flasks. Cells were grown in Dulbecco's Modified Eagle's medium, supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD), 25 mM Hepes, 110 mg/L sodium pyruvate, and 20 mM L-glutamine, in a 95% air:5% CO₂ incubator at 37°C with humid atmosphere.

Vitamin C Stimulation

To analyze the response to ascorbic acid, serum-free medium supplemented with 0.2% lactalbumin hydrolyzate was added to confluent cells and maintained during 24 h. Fresh serum-free media and ascorbic acid in a range from 10 to 200 µg/ml were added to cells, and incubation was maintained for 4–24 additional hours. In each experiment, control flasks without and with vitamin C (29 µg/ml, the physiologic concentration in amniotic fluid [14]) were included. Equimolar amounts of glutathione were included in some flasks to control for the effect of change in the redox potential. After incubation, the cells and the culture media were assayed according to the following techniques.

Vitamin C Quantitation

Ascorbic acid was quantitated at different times (0–4 h) to ensure that vitamin C was present under experimental conditions. Medium and cells were precipitated with 0.35 M perchloric acid, and their filtrates were processed for HPLC. Chromatography was carried out as suggested by Lee et al. [15]. Intracellular ascorbic acid was normalized to 50 µg DNA. Total DNA per well was quantitated according to Burton [16].

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Metalloproteinase Mixed Activity

To evaluate MMP activity in vitamin C-stimulated cells, gelatinolytic activity was quantified using thermally denaturalized radioactively labeled collagen type I/III as a substrate according to Terato et al. [17]. Each sample was assayed in basal conditions and after being treated with 1.0 mM aminophenol mercuric acetate, an MMP activator. MMP activity was calculated taking into account the EDTA-inhibitable activity and was expressed as specific activity (μg degraded gelatin/ μg incubated protein per 12 h at 37°C).

Gel-Substrate Gelatinolytic Activity

Ten micrograms of protein from vitamin C-conditioned media was applied per lane to a gelatin-containing polyacrylamide gel in small format (Bio-Rad, Richmond, CA), prepared according to a previously described method [12]. At the end of the process, gels were stained with Coomassie R-250 blue. Molecular weight markers (M_r 14–200) were included on each run. To evaluate the direct effect of vitamin C on MMP activity, pro-MMP-2 was purified as suggested by Murphy and Crabbe [18].

Western Blotting

Ten micrograms of protein from conditioned media per lane was applied to a 10% SDS-polyacrylamide gel under nonreducing conditions. Protein electrotransference to Immobilon-P membranes (Millipore, Medford, MA) using a semi-dry system was performed according to Towbin et al. [19]. Membrane was developed with monospecific polyclonal antibodies against 72-kDa type IV collagenase (MMP-2), generously donated by Dr. William Stetler-Stevenson from National Institute of Cancer (NIH, Bethesda, MD) [20]. Primary antibodies were detected with the ABC method (Vector Labs., Burlingame, CA).

Northern Blot

Total RNA from cells subjected to various ascorbic acid concentrations was extracted using Trizol (Gibco BRL). RNA was run in agarose gels and transferred to Immobilon-N membranes (Millipore). For collagen mRNA detection, poly-A was selected using oligo-dT cellulose according to previously described techniques [21]. Membranes were hybridized with probes for MMP-2 [22], type I collagen [23], and β -actin. The probes were labeled with the random primer method using [^{32}P]CTP. Autoradiographies were quantitated with an EagleEye image analyzer (Stratagene, La Jolla, CA). The relative expression was normalized with β -actin mRNA.

Run-On Assay

WISH cells were grown in 25-cm² culture flasks until 70% confluence; then fresh medium containing vitamin C was added as mentioned above. Nuclei from these cells were purified, and the newly transcribed RNA were labeled with [^{32}P]UTP according to Davis et al. [24]. DNA probes for MMP-2 and β -actin were transferred and cross-linked to Z-probe membranes (Bio-Rad) using a slot-blot apparatus. Labeled RNA were hybridized to immobilized probes and developed by autoradiography. The transcription rate was calculated by comparing the MMP-2 spots with the β -actin spots.

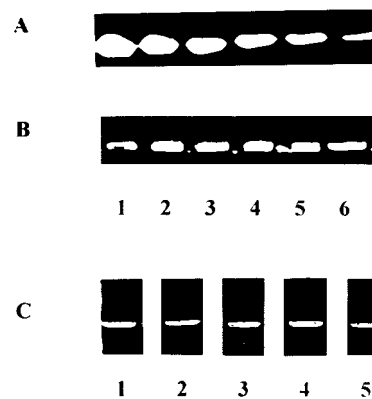


FIG. 1. Gelatin substrate gels. Lanes 1–5 correspond to the gelatinolytic activity in media of cells stimulated with 0, 10, 29, 100, and 200 $\mu\text{g}/\text{ml}$ of vitamin C (A) or equimolar amounts of glutathione (B). Purified pro-MMP-2 standard is in lane 6. Progressive decrease of activity was detected in vitamin C-treated cells. Purified pro-MMP-2 was loaded in five lanes; once run, individual lanes were excised and incubated in a buffer to which vitamin C had been added in the same concentrations as mentioned above. No direct effect of vitamin C on enzymatic activity was documented (C).

RESULTS

Media from WISH cells incubated in the absence of vitamin C showed a gelatinase-specific activity (mean \pm SD, $n = 8$) of 124.3 ± 4.9 μg degraded gelatin/ μg protein. Gelatinolytic-specific activity decreased progressively with 10 μg of vitamin C to 82.1 ± 9.7 , with 29 μg to 63.0 ± 7.8 , with 50 μg to 52.6 ± 9.6 , with 100 μg to 47.8 ± 6.1 , and with 200 μg to 37.2 ± 6.1 . All concentrations of vitamin C significantly (ANOVA, $p < 0.01$) diminished the gelatinolytic activity considering the absence of vitamin C as the basal production. A 4-h incubation in the presence of vitamin C was enough to obtain a maximum effect (data not shown). Concentration of intracellular vitamin C during this time was assessed. Most of the ascorbic acid (86%, range from 83.0 to 88.3, $n = 8$) remained in the extracellular space under experimental conditions.

Gel-substrate assays of the WISH media showed the presence of a main lysis band with an estimated molecular mass of 71 kDa. This band corresponded to the MMP-2 or gelatinase A, according to the relative migration of the purified standard. This lysis band decreased its relative intensity as the vitamin C dose increased (Fig. 1A). Equimolar amounts of glutathione did not affect the basal expression of MMP-2 (Fig. 1B). According to semiquantitative densitometric analysis and taking the vitamin C-free cell media as 100%, residual activity was approximately 60% in the 29 $\mu\text{g}/\text{ml}$ dose (range from 53% to 72%, $n = 8$) and 18% in the 200 $\mu\text{g}/\text{ml}$ vitamin C dose (range from 15% to 30%, $n = 8$). ANOVA on ranks showed differences between all treatments ($p < 0.0001$). Ascorbic acid did not have a direct effect on enzymatic activity as demonstrated by the gel-substrate assay of purified human proenzyme 72-kDa type IV collagenase in the presence of equivalent amounts of ascorbic acid (Fig. 1C).

Western blotting of all the assayed media revealed the presence of a 71-kDa molecular mass band detected with the antibody directed against MMP-2. The relative intensity of this band decreased to 72% with 29 $\mu\text{g}/\text{ml}$ (range from 70% to 78%, $n = 8$) and to 55.6% with 200 $\mu\text{g}/\text{ml}$ of vitamin C (range from 45.0% to 65.1%, $n = 5$) compared to that for the cells incubated in absence of vitamin C (Fig.

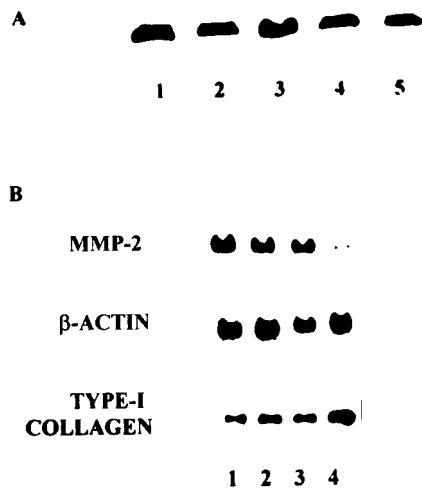


FIG. 2. Western and Northern blot. **A)** Immunoreactive MMP-2 decreased in the media of vitamin C-stimulated WISH cells. Lanes 1–5 contained 0, 10, 29, 100, and 200 $\mu\text{g/ml}$ of vitamin C, respectively. **B)** Basal levels of MMP-2 mRNA (lane 1) decreased in the cells stimulated with 29, 100, and 200 $\mu\text{g/ml}$ of vitamin C (lanes 2–4). No effect was observed in β -actin mRNA, and the opposite effect was documented in type I collagen mRNA.

2A). ANOVA on ranks revealed significant differences between all groups ($p < 0.001$).

MMP-2 mRNA showed a diminished expression when the concentration of vitamin C increased (Fig. 2B). Relative densitometric intensity decreased to 63% with 29 $\mu\text{g/ml}$ of vitamin C (range from 58% to 72%, $n = 4$) and to 8.7% with 200 $\mu\text{g/ml}$ (range from 6% to 11.0%, $n = 4$). Statistical differences reached $p < 0.0001$ between groups. In contrast, intensity of bands for type I collagen increased in the opposite way. The band of β -actin did not change in the presence of different concentrations of vitamin C (Fig. 2B).

Transcription rate for MMP-2, as measured by relative densitometric intensity in the run-on assays, decreased to 13% (range 11% to 17%, $n = 3$) in those nuclei obtained from cells stimulated with 200 $\mu\text{g/ml}$ of vitamin C as compared to the basal rate in the absence of the vitamin (Fig. 3).

DISCUSSION

Type I, type III, and type IV collagens are the main structural constituents of chorioamniotic membranes, and

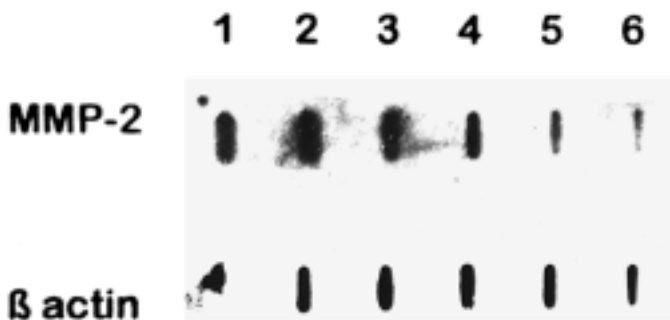


FIG. 3. Run-on assay. WISH cells were stimulated with 0, 10, 29, 50, 100, and 200 $\mu\text{g/ml}$ of vitamin C (lanes 1–6), and the newly synthesized labeled RNA were hybridized against membrane-linked probes for β -actin and MMP-2. A progressive decrease in the transcriptional rate of the MMP-2 mRNA depending on the vitamin C dose was observed.

they are responsible for the strength and flexibility of the membranes. In this study, it was demonstrated that vitamin C, in addition to its well-known role in collagen biosynthesis, modulates the degradation of this protein. The regulatory effect of vitamin C is exerted on the 72-kDa type IV collagenase or MMP-2 gene expression. MMP-2 is the only MMP secreted by WISH cells under experimental conditions. This peculiarity makes WISH cells a good model for study of MMP synthesis and secretion in chorioamnion during gestation. The unique presence of MMP-2 in amniotic fluid and fetal membranes during midgestation suggests its role in extracellular matrix remodeling in chorioamnion [12, 13, 25]. MMP-2 degrades type IV collagen, which is found in association with basal membranes but in chorioamnion is very widespread in a network [26].

Vitamin C was carried by cultured cells under experimental conditions, and even with the low intracellular concentration, the effect on MMP-2 expression was demonstrated. The slow cellular intake may be explained by the fetal calf serum-free conditions that are necessary for the MMP-2 enzymatic activity assays. Fetal calf serum is contaminated with variable amounts of vitamin C and several MMPs that mask the effect on MMP-2 expression, as reported previously [27]. Vitamin C did not directly deactivate MMP-2 but had an effect on MMP-2 genomic expression as revealed by the Western blots, Northern blots, and run-on assays. Down-regulation of MMP-2 gene expression by vitamin C is exerted at physiologic concentrations if we consider the concentrations in amniotic fluid a good indicator of what is occurring inside the chorioamnion. A nonspecific effect of vitamin C on gene expression, mediated by changes in the cellular redox potential, may be overruled by the lack of effect of equimolar amounts of glutathione and the parallel observations of no change in β -actin gene expression and increase in type I collagen gene expression.

A model of the effect of vitamin C on extracellular matrix remodeling of the chorioamnion during pregnancy may be proposed: if there is low availability of vitamin C in pregnancy, an increase in activity of MMP-2 may occur, leading to increased collagen degradation and a cumulative loss of the main support of the chorioamnion resulting clinically in PROM. We cannot rule out the possibility that collagen synthesized under low availability of vitamin C has a low hydroxyproline and hydroxylysine content and this means that collagen is intrinsically less resistant. The low mechanical strength of PROM-derived chorioamnion may be understood in terms of a simultaneous effect of the above-mentioned factors.

PROM has been linked to conditions that prevail in developing countries and that are associated with a low socioeconomic level. A vitamin C-deficient diet during pregnancy could be one explanation for why PROM is more frequent in these countries. In addition, these populations are characterized by increased prevalence of infections, which in turn may induce an extra consumption of ascorbic acid. To validate the role of vitamin C in PROM it is necessary to implement other studies that will complement the significance of these results at levels other than the molecular level.

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