Resting Chondrocytes in Culture Survive without Growth Factors, but Are Sensitive to Toxic Oxygen Metabolites

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Abstract. Chondrocytes in dense suspension culture in agarose survive in serum-free DME because they secrete low molecular mass compounds supporting their own viability. This activity can be replaced by pyruvate, or sulfhydryl compounds, e.g., cysteine or dithioerythritol. Catalase, an enzyme decomposing H_2O_2, also protects the cells, whereas superoxide dismutase has no effect. Therefore, chondrocytes in culture are sensitive to toxic compounds derived from molecular oxygen, i.e., hydroxyl radicals or hydrogen peroxide spontaneously generated in DME containing ascorbate and ferrous ions. Poly-ADP-ribosylation is an important step in the cascade of events triggered by these compounds.

To survive, chondrocytes do not require stimulation by growth factors. They remain resting cells in fully defined, serum-free culture also at low density. Proliferation and hypertrophy can be induced by serum, but not by low cell density alone.

During respiration, cells reduce large amounts of molecular oxygen to water, but minor quantities of H_2O_2 and $O_2^{-\cdot}$ or $OH^{\cdot}$ radicals are also formed. Aerobic cells protect themselves against these toxic oxygen species by decomposing them with superoxide dismutases, catalase, and/or peroxidases or with antioxidants, such as α-tocopherol, carotinoids, bilirubin, pyruvate, glutathione, or cysteine (for review, see Cadenas, 1989).

Hyaline cartilage is an avascular tissue with relatively few cells embedded in abundant extracellular matrix. Therefore, chondrocyte metabolism and differentiation depends on diffusion of nutrients and regulatory factors, either from surrounding tissues or from one cartilage cell to another. Cartilage matrix is penetrated even by large molecules because it consists of a loose network of collagen fibrils entrapping the highly charged, polyanionic proteoglycans which bind large amounts of water (Maroudas and Bannon, 1981). When compared with cells of vascularized tissues, chondrocytes in situ are exposed to very low oxygen tensions (Maroudas, 1973; Stockwell, 1983). It is conceivable, therefore, that they are exceptionally sensitive to oxygen, particularly at pressures normally encountered in tissue culture. In fact, development of the chondrocytic phenotype by mesenchymal cells in culture is favored by reduced oxygen pressures (Pawelek, 1969).

Chick embryo sternal chondrocytes in suspension culture survive even in the absence of serum when grown at high density. The conditioned media support the viability of similar cells at low density suggesting that chondrocytes produce factors required for their own survival (Bruckner et al., 1989). Here, we identify this activity as a low molecular weight antioxidant and as the only prerequisite for cell viability.

Materials and Methods

Agarose Cultures

Chick embryo sternal chondrocytes were cultured in agarose at densities between 0.08 and 1.0 × 10^6 cells/ml (Benya and Shaffer, 1982; Bruckner et al., 1989). Briefly, 60-mm Petri dishes were coated with agarose and cell suspensions in low melting agarose were overlayered. To facilitate their observation, cells were sedimented at 37°C to the interface between the agarose layers. The low melting agarose was then allowed to gel by brief exposure of the cultures to 4°C. Cultures were supplied with appropriate media which were regularly replaced after 48 h.

Culture Media

The culture media were based on serum-free DME, containing 60 μg/ml of β-aminopropionitrile fumarate (Fluka AG, Buchs, Switzerland), and penicillin and streptomycin at 100 U/ml and 100 μg/ml, respectively. Other ingredients were added as specified. Conditioned media were obtained from cultures with high cell density and were subjected to ultrafiltration (model 8MC; Grace Amicon, Wallisellen, Switzerland). The concentrates were diluted with DME to appropriate concentrations. The filtrates were subjected to repeated ultrafiltration on PM 10, YM5, and YM2 membranes, respectively (separation limits: 10, 5, and 2 kD).

Determination of Cell Viability

Whole cultures were stained by adding to 60-mm culture dishes 100 μl of 0.1% (wt/vol) Trypan Blue in PBS. After 10 min, at least five randomly selected micrographs per dish were taken and cells were counted. Cell viability was determined as the fraction of dye excluding cells.

Collagen Synthesis

Cultures were metabolically labeled with radioactive proline. The collagens were extracted after pepsin digestion of the cultures and were analyzed as described (Bruckner et al., 1989).

Results

Primary chondrocytes of chick embryo sterna are viable at a density of 2×10^7 cells/ml in agarose gels in DME containing 50 μg/ml of ascorbate, but no growth factors or serum. Media conditioned by such cultures support viability of cells at lower densities. Upon ultrafiltration, the compound was not retained in the concentrate, even when the molecular mass cut-off of the membranes was 2 kD. However, it bound to mixed bed ion exchange columns and was extractable, albeit in small quantities, into ethyl acetate independently of the pH of the aqueous phase. Therefore, the activity probably is not due to a known growth factor but rather resides in small, possibly zwitterionic molecules.

Ishikawa et al. (1985) reported that chondrocytes in culture produce ammonia and several amino acids. Therefore, culture media were supplemented with 1 mM of Asn, Asp, Glu, Ala, Cys, Arg, and NH_4Cl, respectively. Only in media containing cysteine were the cells viable for at least 2 wk (Fig. 1). Survival was dose dependent, optimally required 1-2 mM of cysteine, and clearly decreased above 5 mM (Fig. 2).

To determine whether the sulfhydryl group of cysteine was relevant rather than its importance as an amino acid, we replaced cysteine in the culture media by dithioerythrol. Results almost identical to those shown in Figs. 1 and 2 were obtained (not shown), demonstrating the importance of the sulfhydryl groups.

Since sulfhydryl reagents can act as oxygen radical scavengers (Cadenas, 1989), these observations suggested such radicals to be a possible cause of the observed cytotoxicity. Hydrogen peroxide is known to suppress proteoglycan production in cartilage or chondrocytes in culture (Bates et al., 1985; Schalkwijk et al., 1985), or to reduce the rate of proliferation of chondrocytes in monolayer culture (Vincent et al., 1989). Oxygen radicals arise from molecular oxygen by one-electron transfer reactions. For example, $O_2^{-\cdot}$ and $OH^{\cdot}$ can be formed in the presence of Fe^{2+} according to:

$$O_2 + Fe^{2+} \rightarrow O_2^{-\cdot} + Fe^{3+} \quad (1)$$
$$2O_2^{-\cdot} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (2)$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+} \quad (3)$$

Ascorbate can also serve as a source of electrons in the generation of toxic oxygen metabolites. In agreement with this concept, omission of ascorbate from DME resulted in improved cell viability (Fig. 3).

Toxic oxygen-derived compounds can be eliminated enzymatically. $O_2^{-\cdot}$ is decomposed by superoxide dismutase, and catalase degrades H_2O_2 to oxygen and water, thus also preventing the production of $OH^{\cdot}$ (formula 3). Both of these enzymes are known to protect cells from oxygen toxicity (Cadenas, 1989). When superoxide dismutase was added to our culture media, cell viability was not improved whereas catalase clearly protected the cells. Superoxide dismutase also had no effect in conjunction with catalase (Fig. 4). These results show that oxygen toxicity caused the loss of viable cells in serum-free chondrocyte culture. The findings also emphasize the importance of H_2O_2 or $OH^{\cdot}$, rather than $O_2^{-\cdot}$. Moreover, the toxic compounds appeared to originate from the media rather than intracellularly.

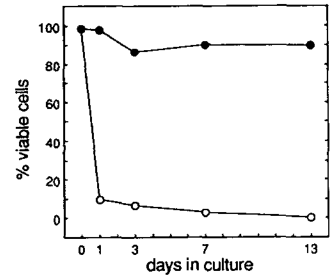

Figure 1. Support of chondrocyte survival by cysteine in agarose culture. Cells were plated at a density of 2×10^6 cells/ml and were supplied with DME containing (–●–), or lacking (–○–), 1 mM of cysteine.

Andrae et al. (1985) and O'Donnell-Tormey et al. (1987) have shown that mammalian cells in culture secreted pyruvate and related α-ketoacids to protect themselves against hydrogen peroxide-induced cytotoxicity. When cultures without cysteine were supplied with pyruvate, cells survived to an extent similar to that observed with cysteine (Fig. 5). Glucose is available to the cells at a large excess. Therefore, the intracellular pool of pyruvate probably is not affected by 1 mM of exogenous pyruvate indicating again that hydrogen peroxide toxicity was derived from the media.

Berger (1985) proposed that the toxic effects of hyperoxia and hydrogen peroxide are indirectly mediated by "suicidal" poly-ADP-ribosylation of intracellular proteins with concomitant depletion of the cellular NAD^+ pools. If this mechanism is operative, cell viability ought to be improved by specific inhibitors of poly-(ADP-ribose)polymerase, e.g., benzamide or 3-aminobenzamide. Gille et al. (1989) have shown that certain cell types were protected by 3-aminobenzamide while others were not. Chondrocytes in our culture system, however, were protected by 0.5 μg/ml of benzamide documenting the low toxicity of this drug. However, long term viability was not supported by the drug alone, but also required the presence of cysteine (Fig. 5). These observations indicated that the effects of toxic oxygen species were not entirely abolished by benzamide implying that, under our conditions, poly-ADP-ribosylation was an important, but not the only, cause of cell loss. Interestingly, Baker et al. (1989) reported that ATP levels were suppressed in chondrocytes exposed to H_2O_2 because of oxidation of glycolytic enzymes.

In serum-free culture, chondrocytes did not proliferate (Table I). However, after 7 d in culture, the cells produced radioactively labeled collagens II, IX, and XI, whereas collagen X, a marker for hypertrophic chondrocytes, was not detectable (Fig. 6, lanes *1* and *3*). Therefore, the cells resem-

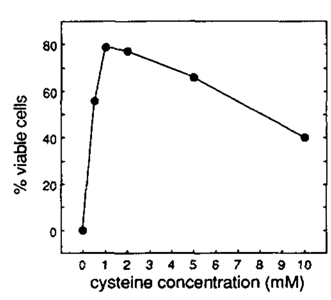

Figure 2. Cysteine dose dependence of survival of chondrocytes in agarose suspension. Cells were cultured as in Fig. 1 and the media contained cysteine at the concentrations indicated.

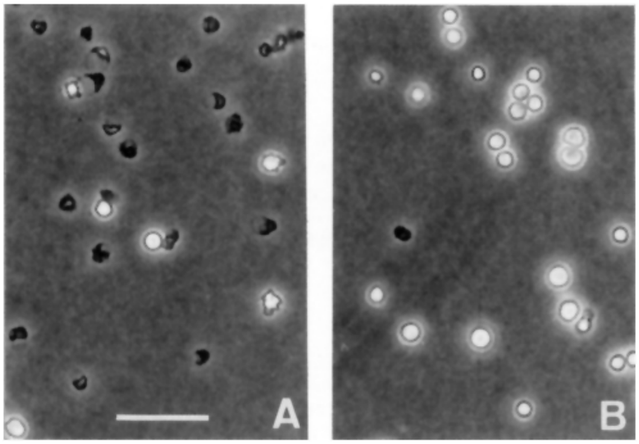

Figure 3. Phase-contrast micrographs of chondrocytes grown in media with 50 μg/ml (*A*) or without (*B*) ascorbate. Micrographs were taken after 1 d in culture. Bar, 50 μm.

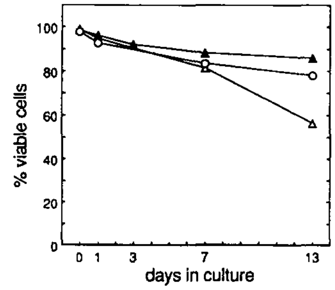

Figure 5. Chondrocyte viability in agarose culture in media containing pyruvate or benzamide. The media contained 1 mM of pyruvate (–○–), 500 nM of benzamide (–△–), or 500 nM of benzamide and 1 mM of cysteine (–▲–).

bled resting chondrocytes. When FBS was added to the media, the cells became hypertrophic and secreted large amounts of collagen X (Fig. 6, compare lanes *1* and *3* with lanes *2* and *4*). In agreement with our recent results (Bruckner et al., 1989), this observation demonstrated that FBS could induce in vitro chondrocyte hypertrophy that was not prevented by antioxidants.

Discussion

The results presented here support two major conclusions. Firstly, chondrocytes in agarose suspension culture are sensitive to oxygen-derived toxicity arising in the culture media. The cells are effectively protected by antioxidants which are either supplied exogenously or produced by the cells themselves. Likely candidates for cellular products are glutathione or cysteine (Cadenas, 1989). Cellular NAD^+ depletion following poly-ADP-ribosylation has tentatively been identified as a step in the sequence of events leading to cell death.

The high sensitivity of chondrocytes towards oxygen toxicity is plausible in view of the low oxygen pressures in cartilage and highlights possible mechanisms of tissue destruction in degenerative cartilage disease. During phagocytosis, inflammatory cells actively produce oxygen-derived radicals as a means to eliminate infectious agents. As a consequence, cartilage degeneration is induced either by direct damage or by stimulation of the production of proteolytic enzymes, including collagenases (Burkhardt et al., 1986).

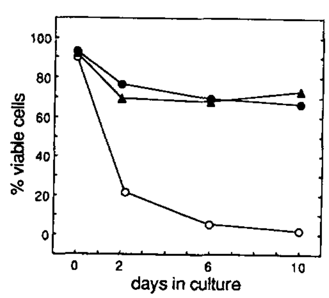

Figure 4. Chondrocyte viability in agarose culture in media containing catalase and superoxide dismutase. Cells were cultured in DME lacking cysteine, but containing catalase (–●–), 1 μg/ml of superoxide dismutase (–○–), or both enzymes (–▲–).

Our second conclusion concerns the growth factor requirements of resting chondrocytes. Because of their dependence on growth promoting activities, most cells in culture routinely are supplied with sera or other undefined additives. Some cells also require specific growth factors. For example, epidermal cells in culture only survive in the presence of EGF (Tsao et al., 1982) and nerve cells depend on nerve growth factor in addition to other, less well-defined components (Barde et al., 1983). Cartilage cells and their precursors also respond sensitively to hormones or growth factors, e.g., by proliferation, differentiation, or production of collagens and proteinases (Pawelek, 1969; Tyler, 1985; Lindahl et al., 1987; Mauviel et al., 1988; Reddini et al., 1988; Andrews et al., 1989). However, the results presented here show that the viability of resting chondrocytes strikingly

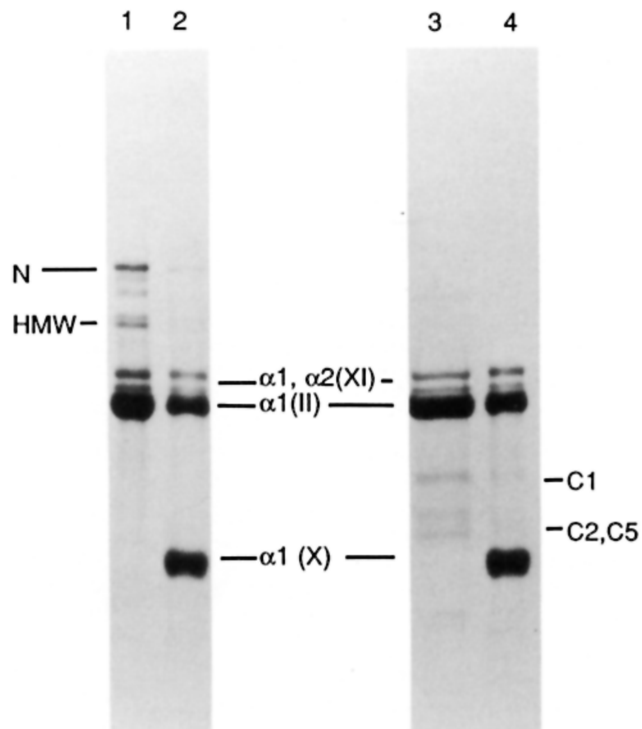

Figure 6. SDS-PAGE (4.5–15% gradient gel) of pepsin-treated collagens produced by chondrocytes (2 × 10^6 cells/ml) after 7 d in culture. A fluorogram is shown of newly synthesized collagens radiolabeled with [^{14}C]proline. The media without (lanes *1* and *3*) or with 10% of FBS (lanes *2* and *4*) contained 1 mM of cysteine. Samples in lanes *3* and *4* were reduced with 2% 2-mercaptoethanol before electrophoresis. *N* and *HMW* are pepsin fragments of collagen IX; *C1*, *C2*, and *C5* are constituent polypeptides of *HMW*.

Table I. Proliferation of Chick Embryo Chondrocytes in Serum-Free DME, 1 mM Cys

	Cells/ml agarose ($\times 10^{-6}$) at day				
Experiment	0	1	3	7	13
1	1.00 ± 0.10	0.60 ± 0.09	0.70 ± 0.04	0.70 ± 0.09	0.60 ± 0.06
2	0.40 ± 0.06	0.40 ± 0.03	0.40 ± 0.09	0.30 ± 0.05	0.30 ± 0.07
3	0.08 ± 0.01	0.10 ± 0.02	0.07 ± 0.01	ND	0.05 ± 0.01

Whole cultures were stained with trypan blue and representative light micrographs were taken. The number of viable cells was determined in four micrographs per culture dish.

does not depend on stimulation by hormones or growth factors.

Hypertrophy of chondrocytes at low density in agarose cultures is induced by FBS. However, it remains possible that the low cell density alone is responsible for this effect. Here, we show that resting cells can be maintained in serum-free culture also at low density. This implies that serum components are required for the late stages of chondrocyte differentiation.

Finally, the culture system described here offers advantages for studies on the regulation of chondrocyte differentiation. The culture media are fully defined and no conditioned media are necessary. The system is superior also to the one we have developed recently because the low cell density allows smaller amounts of regulation factors to be effective.

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