Antioxidant enzymatic activities and lipid peroxidation in cultured human chondrocytes from vertebral plate cartilage

Vittorio Grazioli[a,*], Roberta Schiavo[a], Erminia Casari[a], Fulvio Marzatico[b], Riccardo Rodriguez y Baena[c], Paolo Gaetani[c]

[a] *Department of Laboratory Medicine, Istituto Clinico Humanitas, 20089 Rozzano, Italy*
[b] *Neurosurgery Unit, Istituto Clinico Humanitas, 20089 Rozzano, Italy*
[c] *Institute of Pharmacology, University of Pavia, Pavia, Italy*

Received 25 May 1998; revised version received 10 June 1998

Abstract In vitro models based on primary cultured human chondrocytes could be useful to study the ROS-mediated inflammatory processes that seem to involve chondrocytes in vivo. In this work, we studied the enzymatic antioxidative capability of human chondrocytes removed from vertebral plates during micro-discectomy and cultured 18 days, measuring total superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) activities. We also evaluated in the same cells the amount of malondialdehyde (MDA) in order to verify the effect of the variation of the cellular enzymatic antioxidative capability on the degree of membrane lipid peroxidation.
Total SOD activity increased, even if not significantly, between the 12th and the 18th day. A significant variation of GSHPx ($P < 0.01$) and of catalase ($P < 0.001$) activity was observed between the 3rd and the 6th day with no further variation until the 18th day. A significant increase ($P < 0.001$) of lipid peroxidation from the 3rd to the 18th day was also observed. These results seem to indicate that only fresh human cultured chondrocytes are suitable to study, through in vitro models, the in vivo behavior of the antioxidative status of these cells.

Key words: Human chondrocyte; ROS; Superoxide dismutase; Catalase; Glutathione peroxidase

1. Introduction

Although it is widely accepted that disc compression does not induce pain per se [1,2], it was only recently demonstrated that in the functional changes that occur in compressed nerve roots phlogistic processes may be involved [3–6]. In particular, Olmaker et al. [3], by mean of in vivo models based on the use of pig cauda equina, found that the biological effects induced by the herniated material is related to a viable cellular population. Since chondrocytes represent the main cellular components of both intervertebral disc and its contiguous vertebral plate cartilage, it is likely to assume that they might be involved in inflammatory processes also at this level.

In effect, several works showed that chondrocytes are actively involved in inflammation processes, particularly in the induction and modulation of the pathways that under pathologic conditions link inflammatory mediators and proteases [7–13].

Particularly, little is known about the relationship that links reactive oxygen species and human chondrocytes in the inflammatory damage, although it is already known that also ROS can be involved in inflammatory processes [14]. Recently, Shingu [12] demonstrated that IL-6 inhibits superoxide production by chondrocytes and suggested that oxygen radicals could be involved in the cartilage matrix degradation, mediating the release of collagenase by chondrocytes.

On the basis of these considerations, primary cultures of human chondrocytes from discal and vertebral plate cartilage appear to represent a useful in vitro model to assess the oxidative/antioxidative status of human chondrocytes and study ROS-mediated epidural inflammatory process in vivo.

In this work, we studied the enzymatic antioxidative capacity of human chondrocytes derived from vertebral plates under micro-discectomy and cultured for 18 days, and measured total superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) activities. We also determined the amount of malondialdehyde (MDA) in the same cells in order to verify the possible correlation between the cellular enzymatic antioxidative capacity and the degree of membrane lipid peroxidation.

2. Materials and methods

2.1. Chemicals and other reagents

Ca^{2+} and Mg^{2+} free PBS solution, Ham's F-12 medium, penicillin-streptomycin stabilized solution (10 000 U and 10 mg/ml respectively), trypsin solution (0.25% in HBSS), solid crude type IA collagenase (≥125 collagen digestion units per mg), FCS, L-ascorbic acid sodium salt, L-glutamine solution (200 mM), trypsin-EDTA solution (1% trypsin and 0.05% EDTA), trypan blue 0.4% solution, SOD standard, $KMnO_4$, H_2SO_4, H_2O_2, ethanol, Triton X-100 and reduced glutathione were purchased from Sigma Chemicals (USA).

Cytochrome *c*, xanthine, xanthine oxidase, glutathione reductase and NADPH were purchased from Fluka (Switzerland).

Chondrocyte cultures were carried out on 1 cm^2 flat bottom cuvettes (Multiwell, 24 well Falcon 3047, Becton Dickinson, USA).

2.2. Tissue sampling and preparation

Since obvious ethical reasons do not allow to draw vertebral plate cartilage from living healthy subjects, we obtained the material to study from 30 patients (23 males and 7 females, mean age was 41.2 ± 6 years) admitted at our Institution for surgical treatment of disc disease. Neurological examination before surgery showed in all cases an history of lumbar and sciatic pain with radicular distribution for at least 4 months and, at admission, sensory and/or motor root involvement. Radiological investigations (CT scan and/or MRI) showed in all cases a disc herniation (20 protruded discs and 10 disc extrusions). In eight cases the level of disc herniation was L4-L5 and in 12 cases L5-S1. None of the patients received in the three weeks preceding surgery corticosteroid therapy. Informed consent was

*Corresponding author. Fax: +39 (2) 8224.4790.
E-mail: vittorio.grazioli@humanitas.it

Abbreviations: ROS, reactive oxygen species; PBS, phosphate buffered saline; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; LDH, lactate dehydrogenase; GSSG, glutathione (oxidized form); SOD, total superoxide dismutase; CAT, catalase; GSHPx, glutathione peroxidase

PII: S0014-5793(98)00731-5

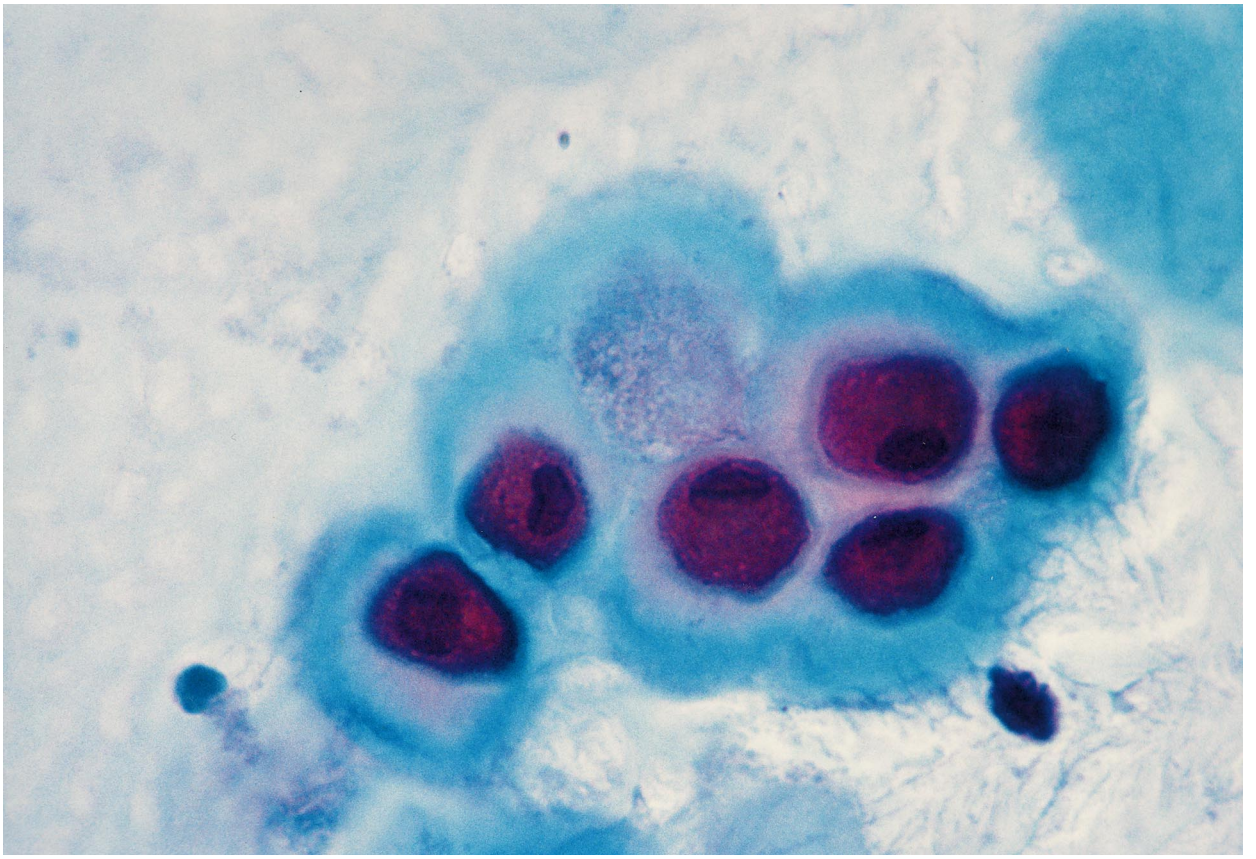

Fig. 1. Human chondrocytes in culture 18 days after seeding (Papanicolaou staining, magnification ×1000). For details see Section 2.

signed by all patients in the last 20 days before the intervention. Surgery was in all cases performed in general anesthesia (Propofol I.V. or Halotane in gas mixture) and, after microsurgical decompression of disc space with removal of herniated material, specimens (100 to 500 mg) of macroscopically normal, neither degenerated nor inflammated, cartilage were removed from lumbar vertebral plates with micro-curettes, as standard operative procedure. The specimens were immediately washed thoroughly with 9% NaCl sterile solution to remove all blood and bodily fluid contamination, then put into sterile tubes, sent to the laboratory and immediately processed.

2.3. Chondrocytes isolation and culture

Human chondrocytes were isolated according to the method described by Grover et al. [15] and by Shingu et al. [12] with some modifications. Briefly, after washing twice with PBS, cartilage was dipped in Ham's F-12 medium with 1% v/v penicillin-streptomycin solution and then divided into small fragments which were maintained with 5 ml of 0.25% trypsin HBSS solution for 30 min in a 5% CO_2/95% air, humidified incubator set at 37°C. After incubation, the cartilaginous fragments were washed twice with PBS and digested overnight with 0.05% (w/v) collagenase solution in culture medium. After digestion, the remaining tissue pieces were removed by filtration and the chondrocyte suspension was washed twice with PBS, centrifuged (1500×*g*, 5 min) and the pellets were re-suspended in 1 ml of Ham's F-12 medium. After evaluation of the cellular viability (see Section 2.4), chondrocytes were seeded at a density of 2×10^3 cells/cm^2 and maintained in culture with Ham's F-12 medium supplemented with 10% FCS, 5% ascorbic acid, 0.5% L-glutamine and 1% penicillin-streptomycin solution) for 18 days at 37°C. Culture medium was changed twice a week.

Cellular homogeneity was evaluated microscopically immediately after seeding and after 3, 6 and 18 days of culture (Fig. 1).

2.4. Biochemical analyses

As a marker of cellular specificity and de-differentiation which occurs with time in culture, proteoglycans produced and released from the cells were determined (see below) in conditioned medium sampled randomly from different wells at 3, 6, 12 and 18 days after seeding. In the same samples, but also in the conditioned medium of the first 24 h of culture, LDH activity was determined (see below) in order to indirectly estimate possible cell damage. The remaining viable cells recovered from the same wells were counted microscopically in a Burker chamber after trypan blue incorporation to evaluate both cell number (growth) and mortality.

The antioxidant capability of cultured chondrocytes was evaluated directly, by measuring specific enzymatic activities, and indirectly, by determining the oxidative damage at the level of cellular membrane. To this purpose, chondrocytes recovered from eight different wells, randomly chosen at 3, 6, 12 and 18 days of culture, were suspended in 3 ml of Ham's F-12 medium and sonicated (1 min; 0.09 W). The obtained suspension was divided into 1 ml aliquots, for malondialdehyde (MDA) determination, and 2 ml aliquots (that were further centrifuged, 4000×*g*, 20 min), in order to measure total superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) activities in the supernatant.

Proteoglycan (PG) concentration was determined by ELISA with a commercially available kit (Biosource Int., USA) used according to the manufacturer's instructions. In particular, for the calibration, standards of Aggrecan purified from articular cartilage supplied with the kit were used and the results were expressed in terms of ng of PG/10^6.

LDH activity was measured at 37°C (LDH, Boehringer Mannheim, Germany) using an automatic analyzer (911 Hitachi, Japan). The results were expressed in terms of IU of LDH/10^6 cells.

Total SOD activity was measured according to the method described by Flohe' and Otting [16], based on the determination of the reduction rate of cytochrome *c* by superoxide radicals, monitored at 550 nm, utilizing the xanthine-xanthine oxidase system as the source of superoxide ions. The results were expressed in terms of U of SOD/10^6 cells, where 1 U of SOD is defined as the amount of

enzyme that causes 50% decrease of the reduction rate of 39.9 nmol cytochrome *c* at pH 7.00 and at 25°C.

CAT activity was determined by titration of residual H_2O_2 with permanganate, according to the technique by Maehly and Chance [17] as modified by Cohen et al. [18]. As the decomposition of H_2O_2 by catalase follows first-order kinetics, the results were expressed in terms of reaction rate constant k/10^6 cells, where k (s^{-1}), under the condition described by the same authors (pH = 7; reaction temperature = 0–2°C; reaction time = 3 min), is directly proportional to the amount of catalase activity [17].

GSHPx activity was measured according to the method described by Flohe' and Gunzler [19], based on the continuous monitoring of NADPH oxidation during the concomitant reduction of GSSG. The results were expressed in terms of mU of GSHPx/10^6 cells, where 1 U of GSHPx is defined as the amount of enzyme necessary to oxidise 1 μmol/NADPH/min at pH 7.0 and at 25°C.

MDA amount was measured using a colorimetric assay for lipid peroxidation (Bioxytech LPO-586, Oxys int., USA). The results were expressed in terms of nmoles of MDA/10^6 cells.

All the analyses were performed in eight samples, each of them in duplicate.

2.5. Statistical analyses

Statistical analysis was performed with one-way analysis of variance (ANOVA).

3. Results

Eighteen days after seeding, cultured cells appeared to be happily alive, homogeneous and near to the confluence (Fig. 1).

Chondrocytes proteoglycan production (Fig. 2a) decreased along the experimental period till reaching a minimum on the 18th day in culture. These results are expected since it is known that these cells in culture go toward a progressive de-differentiation becoming gradually unable to produce proteoglycan and type II collagen [20]. The data obtained from LDH measurement and cell growth monitoring (Fig. 2b and c) indicate that the mortality of the human chondrocytes reached the minimum within the 3rd day of culture, after a significative ($P < 0.001$) decrease from the 1st (29000 ± 1489 UI/10^6 cells) to the 3rd day (9430 ± 252 UI/10^6 cells) corresponding to an expected maximum of initial mortality. From the 3rd to the 18th day the cellular lysis decreased progressively, while the chondrocytes number increased constantly. Fig. 3 summarizes the results obtained by the measurement of the enzymatic activity of SOD (a), GSHPx (b), CAT (c) and MDA amount (d), respectively. The results obtained showed no statistically significant changes of total SOD activity between the 12th and the 18th day (Fig. 2a). Conversely, a significant ($P < 0.01$) variation of GSHPx activity was observed between the 3rd (3.86 ± 0.34 mU/10^6 cells) and the 6th day (2.52 ± 0.26 mU/10^6 cells) with no further variation until the 18th day (Fig. 2b). Catalase activity decreased significantly ($P < 0.001$) from the 3rd (119.5 ± 5.9 K/10^6 cells) to the 6th day (39.6 ± 5.6 K/10^6 cells). From the 6th to the 18th day the enzymatic activity did not vary significantly.

A significant increase ($P < 0.001$) of MDA (as an index of lipid peroxidation) from an initial value of 689.7 ± 113.7 nmoles/10^6 cells (3rd day) to a final value of 2927.8 ± 172.0 nmoles/10^6 cells (18th day) was observed.

4. Discussion

Recently, Olmaker et al. [3] reported that, likely, mediators produced by viable cells are responsible for the functional changes that herniated nucleus pulposus is known to induce on nerve roots through a phlogistic process [4,5]. At the site of lumbar disc herniation, inflammatory cytokines are produced with an increase of prostaglandin E2 production. The subsequent inflammatory status has been experimentally characterized by an in vivo model, as acute sciatica by Sawin et al. [6], as well as an in vitro model [21]. Chondrocytes, which represent the main component of the cell population of intervertebral disc and cartilage, may take part to the inflammatory process.

A link between inflammatory cytokines produced by chondrocytes and reactive oxygen species (ROS) in the pathogenesis of articular inflammation has been recently hypothesized [7–9,12]. In particular, interleukin 1 (IL-1) and tumor necrosis factor α (TNFα) are known to induce an increase of ROS

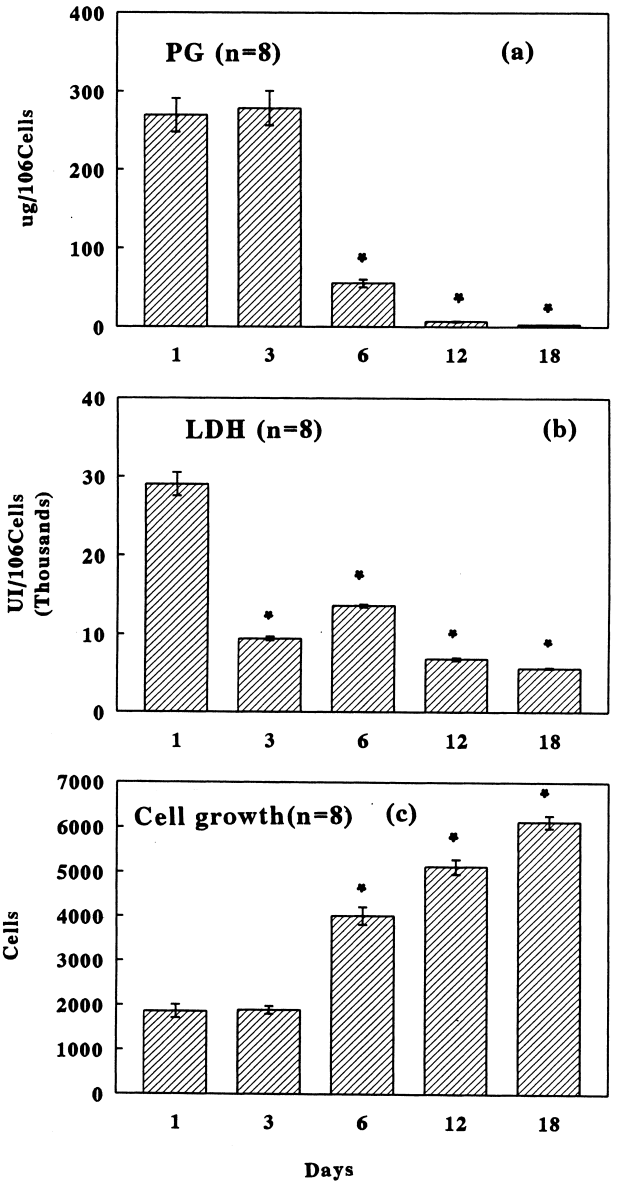

Fig. 2. Changes with culture time of chondrocyte cell number (c) and content of proteoglycans (a) and lactate dehydrogenase (b) in the culture medium. Each bar represents the mean value and the vertical lines show the S.E.M. The symbol * remarks a significant variation ($P < 0.05$).

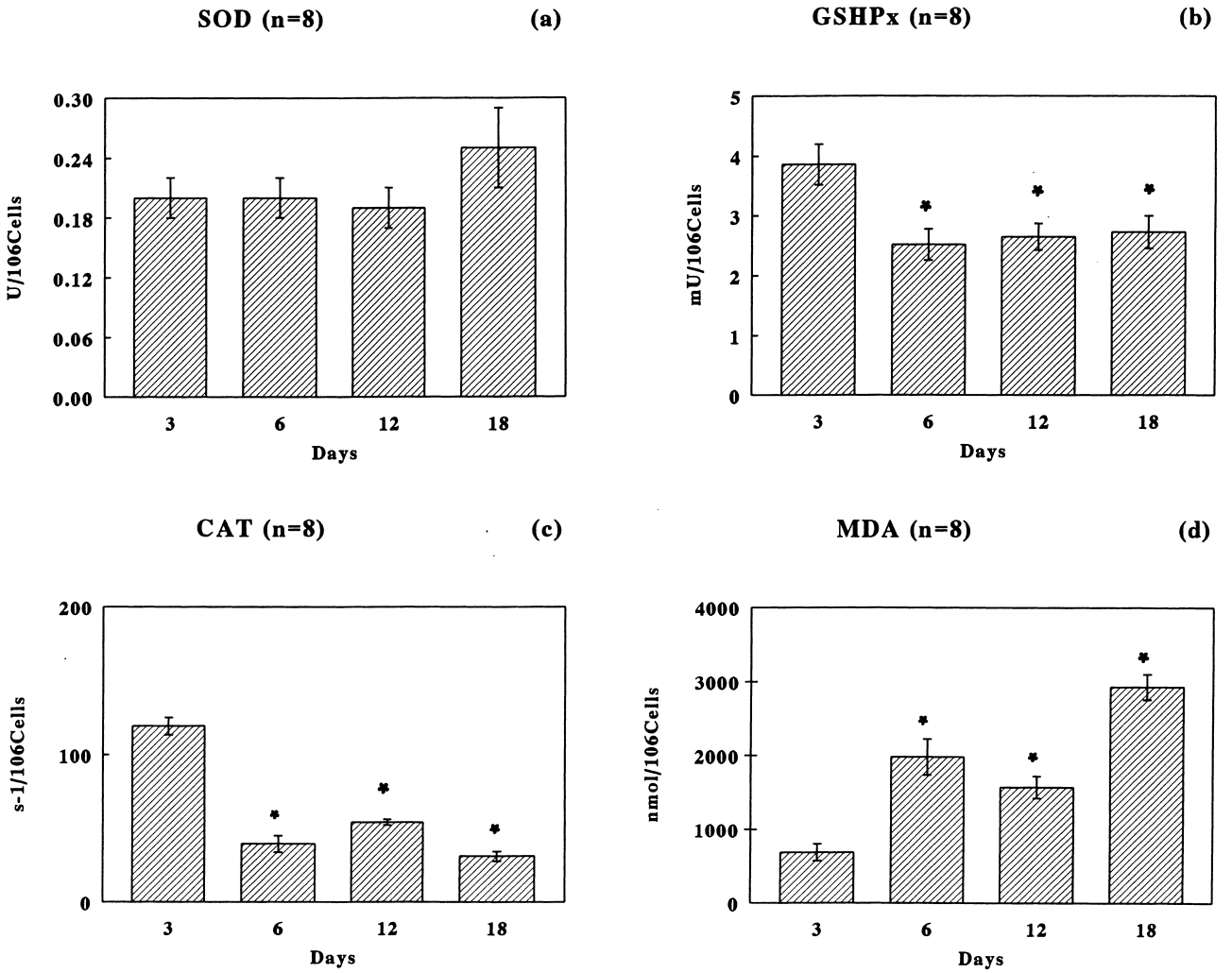

Fig. 3. Variation of malondialdehyde content (d) and superoxide dismutase (a), gluthatione peroxidase (b) and catalase (c) activities during chondrocyte culture. Each bar represents the mean value and the vertical lines show the S.E.M. The symbol * remarks a significant variation ($P < 0.05$).

production by bovine chondrocytes [8]. The oxygen free radicals stimulate the release of a variety of enzymes involved in the degradation of the connective matrix [9,13], like metalloproteinases [11,12]. Furthermore, chondrocytes themselves, as well as other non-phagocytic cells such as endothelial cells [22] and fibroblasts [23,24] can produce superoxide radical O_2^- [12]. In particular, the release of superoxide by chondrocytes NADPH-oxidase-like complexes last much longer than that seen with neutrophils, suggesting that in chondrocytes the potential of ROS release could be very significant [25].

However, despite the knowledge of their role during inflammatory response, very few data are available on human chondrocytes enzymatic antioxidant capacity and lipoperoxidative pattern, both in vivo and in vitro. In particular it was reported that a chronic exposure to H_2O_2 may result in a 5- to 40-fold increase in catalase activity [26] and Henrotin et al. [7] demonstrated that one day old cultured human chondrocytes obtained from different donors show different H_2O_2 production and, thus, different superoxide dismutase and catalase expression.

The results of our work clearly indicate that the de-differentiation that is known to afflict primary cultured human chondrocytes is also characterized by a rapid decrease of their antioxidant enzymatic activity and by a parallel increase of the oxidative cellular damages. Thus, the practical conclusion is that, due to the rapid loss of their original capability in hydrogen peroxide detoxification, only fresh human cultured chondrocytes are suitable to study, through in vitro models, the in vivo behavior of the antioxidative status of these cells. In particular, the absence of a concurrent decrease of SOD activity causes an imbalance of SOD/CAT and SOD/GSHPx ratios with an increase of H_2O_2 availability and, thus, of Fenton's reactions with ROS production. This fact certainly contributes to increase the lipid peroxidation of the cellular membranes whose ageing, in turn, represents a further obstacle to study the mechanisms that (likely through membrane signals) link cytokines, free radicals and degradation of matrix components by proteinases.

References

[1] Howe, J.F., Loeser, J.D. and Calvin, W.H. (1977) Pain 3, (1) 25–41.

[2] RydeviK, B.L., Myers, R.R. and Powell, H.C. (1989) Spine 14, (6) 574–576.
[3] Olmarker, K., Brisby, H., Yabuki, S., Nordborg, C. and Rydevik, B. (1997) Spine 22, (5) 471–475.
[4] Cornefjord, M., Olmarker, K., Rydevik, R. and Nordborg, C. (1996) Eur. Spine J. 5, (3) 187–192.
[5] Olmarker, K., Blomquist, J., Stromberg, J., Nannmrk, U., Thomsen, P. and Rydevik, B. (1995) Spine 20, (6) 665–669.
[6] Sawin, P.D., Traynelis, V.C., Rich, G., Smith, B.A., Maves, T.J., Follett, K.A. and Moore, R.A. (1997) J. Neurosurg. 86, 998–1006.
[7] Henrotin, Y., Deby-Dupont, G., Deby, C., De Bruyn, M., Lamy, M. and Franchimont, P. (1993) Br. J. Reumatol. 32, 562–567.
[8] Lo, Y.Y.C. and Cruz, T.F. (1995) J. Biol. Chem. 270, 11727–11730.
[9] Malfait, A.M., Verbruggen, G., Veys, E.M., Lambert, J., De Ridder, L. and Cornelissen, M. (1994) J. Rheumatol. 21, (2) 314–320.
[10] Lo, Y.Y.C., Wong, J.M.S. and Cruz, T.F. (1996) J. Biol. Chem. 271, 15703–15707.
[11] Campbell, I.K., Golds, E.E., Mort, J.S. and Roughley, J.J. (1986) J. Rheumatol. 13, 20–27.
[12] Shingu, M., Isayama, T., Yasutake, C., Naono, T., Nobunaga, M., Tomari, K., Horie, K. and Goto, Y. (1994) Inflammation 18, (6) 613–623.
[13] Rathakrishnan, C. and Tiku, M.L. (1993) Free Radic. Biol. Med. 15, (2) 143–149.
[14] Halliwell, B. and Gutteridge, J.M.C. (1996) in: B. Halliwell and J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Clarendon Press, Oxford, pp. 422–437.
[15] Grover, J. and Roughley, P.J. (1993) Chem. J. 291, 361–367.
[16] Flohe', L. and Otting, F. (1984) Methods Enzymol. 105, 93–104.
[17] Maehly, A.C. and Chance, B. (1954) in: D. Ghlick (Ed.), Methods of Biochemical Analysis, Vol. 1, Interscience, New York, pp. 357–424.
[18] Cohen, G., Dembiec, D. and Marcus, J. (1970) Anal. Biochem. 34, 30–38.
[19] Flohe', L. and Gunzler, W.A. (1984) Methods Enzymol. 105, 114–121.
[20] Benya, P.D. and Shaffer, J.D. (1982) Cell 30, 215–224.
[21] Takahashi, H., Suguro, T., Okazima, Y., Motegi, M., Okada, Y. and Kakiuchi, T. (1996) Spine 21, (2) 218–224.
[22] Rosen, G.M. and Freeman, B.A. (1984) Proc. Natl. Acad. Sci. USA 81, 7269–7273.
[23] Meier, B., Radeke, H.N., Selle, S., Younes, M., Sies, H., Resch, K. and Habermehl, G.G. (1989) Biochem. J. 263, 539–545.
[24] Meier, B., Cross, A.R., Hancock, S.T., Kaup, F.J. and Jones, O.G.T. (1991) Biochem. J. 275, 241–245.
[25] Tejindevir, S.H., Moulton, P.J. and Hancock, J.T. (1997) Free Radic. Biol. Med. 23, 736–743.
[26] Spitz, D.R., Li, G.C., McCormick, M.L., Sun, Y. and Oberley, L.W. (1988) in: M.G. Simic (Ed.), Oxygen Radicals in Biology and Medicine, Plenum Press, New York, pp. 549–552.