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Mechanism of age-dependent involution in embryonic chick notochords

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Abstract. To study the possible mechanism of the age-dependent involution of the notochord, isolated mesenchyme-free notochords of chick embryos were cultured in vitro and compared with their counterparts in vivo. Two different aspects were evaluated: (1) DNA synthesis measured by [^{3}H]thymidine incorporation and visualized by autoradiography and (2) cell death quantified by counting the number of pyknotic nuclei. The results demonstrate that [^{3}H]thymidine uptake by notochords shows an age-dependent decrease in vitro as well as in vivo. The number of [^{3}H]thymidine-labelled notochord cells, however, is higher in vitro than in vivo. At the same time, there is an age-dependent increase in pyknosis in the notochord in vivo and in vitro. So, during the aging process, the number of both pyknotic nuclei and of [^{3}H]thymidine-labelled nuclei suggest a high turnover of notochord cells in vitro. From these results, we can conclude that the process of involution in aging notochord seems to be controlled by a programmed intrinsic process, which might be influenced partially by the microenvironment in vivo.
Key words. Notochord; [^{3}H]thymidine incorporation; pyknosis; age-dependent involution.

The notochord is a transient embryonic structure with a limited life span of about 10 days in the chick embryo, from the second to the eleventh day of embryonic life. Its transient role is to provide rigidity of the longitudinal axis of the embryo. In addition, it has inductive potential for the formation of cartilage, and it becomes obsolete as soon as the definitive skeleton is formed[5]. According to the staging series of Hamburger and Hamilton[4] remnants of notochord are still visible in chick embryo up to stage 43[8]. During development of the chick embryo, the notochord shows a stage-dependent reduction in DNA synthesis[8] resulting in reduced mitotic activity[5]. This has been demonstrated by a stage-dependent decrease in tritiated thymidine uptake by chick notochord cells. [^{3}H]thymidine ([^{3}H]TdR) uptake by notochord cells, which was high at stages 22 and 27, was markedly lower at stage 31. After that stage, no uptake by the notochord could be visualized by autoradiography[8]. Mitotic figures in the embryonic chick notochord were frequent in the period between stages 6 and 13 and decreased continuously until stage 31[5].

From stage 25 of embryogenesis onwards, notochord cells with pyknotic or karyorrhectic nuclei become more frequent[5], in agreement with the observations of Babic, who reported that during the fetal period the notochord tissue inside the vertebral bodies degenerates in parallel with the process of ossification of the vertebrae[1]. Some authors have hypothesized that an intrinsic process, programmed cell death, might be involved in differentiation[10,11] and termination of the notochord[1]. However, according to others, the scheduled process of progressive cell degeneration may be attributed to progressive metabolic damage due to lack of a blood supply[6,9], suggesting an extrinsic influence.

As the mechanism of involution in this transient structure is not well understood, we considered whether the disappearance of the notochord is regulated intrinsically, or by the influence of the microenvironment in vivo, or by both. Simultaneous observation of proliferation and cell death in vivo versus in vitro might elucidate the mechanism of this age-dependent involution. For this reason, isolated mesenchyme-free notochords in which the in vivo organotypic structure was preserved were cultured in vitro and compared with notochords in vivo during aging. The notochords were studied for their capacity to proliferate at different developmental stages using [^{3}H]TdR incorporation. The process of cell death in aging notochords was evaluated by counting the number of notochord cells with pyknotic nuclei at the same developmental stages.

Materials and methods

To study the mechanism of age-dependent involution in embryonic chick notochord, isolated mesenchyme-free notochords were cultured and compared with their counterparts in vivo by measuring DNA synthesis and by counting pyknotic nuclei.

DNA measurement

Cell proliferation in the notochords was compared, in vitro and in vivo, using [^{3}H]TdR incorporation.

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In ovo. The fertile hen eggs were incubated at 37 °C in groups of 5 for 3.5, 6.5 and 9.5 days, taking the chick embryos to stages 20, 30 and 35[4]. After preliminary experiments, an excess of [^{3}H]TdR was injected into the yolk under the embryo, at a dose of 370 kBq (10 μCi)/100 μl of sterile physiological saline (Ringer's). The embryos were fixed 6 h later in cold (4 °C) glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.4), processed through a series of ascending concentrations of alcohol and acetone, and embedded in epoxy resin [ERL 38216 (SERVA)]. Two-μm-thick sections on gelatin-coated slides were dipped into photographic emulsion (Amersham LM-1) and exposed for autoradiography. After development, the sections were post-stained with 1% toluidine blue for light microscopy. Labelled notochord nuclei were counted in different sections at various levels from five different chick embryos for each stage examined, and expressed as the percentage of the total number of 500 cells per notochord to give the labelling index.

In vitro. The eggs were incubated at 37 °C for 3.5 days, taking the chick embryos to stage 20[4]. Mesenchyme-free notochords of 8 mm length were obtained by trypsinization after microdissection of the chick embryos, and their total DNA content determined according to the method previously described in Ghanem et al.[3]. The notochords were divided into 3 groups and cultured in vitro for 2 h, 3 days and 6 days respectively, making the age of the notochords in vitro comparable to that of the in vivo experiments. The notochords were cultured separately in 35-mm plastic petri dishes. Each dish contained 3 ml of culture medium and was incubated at 37 °C. During in vitro incubation, the culture vessels were shaken gently at 90 rpm on a gyratory shaker to avoid adhesion of the notochords to the bottom of the culture flask. Labelling with [^{3}H]TdR was performed in vitro for 6 h as in the in vivo experiments. The [^{3}H]TdR was added at 370 kBq (10 μCi)/ml of the culture medium (minimum essential medium). At the end of the culture period, the notochords were washed three times, each for 10 min, in sterile physiological saline (Ringer's). The incorporation was quantified in one half of the notochord cultures by liquid scintillation counting. The other half was fixed and prepared for autoradiography.

Liquid scintillation counting. Three groups of five notochords representing the previously described culture groups were solubilized in a lysis buffer [0.1 M NaCl, 40 mM Tris HCl (pH 7), 20 mM EDTA, 0.5% sodium dodecyl sulphate], and the activity was measured with a Packard liquid scintillation spectrometer. The absolute values of scintillation counting were expressed per total DNA content per notochord (cpm/μg) for the different culture groups.

Autoradiography. Five notochords from each of the previously described culture groups were fixed in cold (4 °C) glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.4) and embedded in epoxy resin. Two-μm sections were processed for autoradiography in a way similar to that previously described for the in vivo experiments.

Table 1. [^{3}H]TdR labelling index and pyknotic index of aging notochords in vivo.

Age of notochords (in vivo)	Stage	[^{3}H]TdR labelling index (%)*	Notochord cells with pyknotic nuclei (%)*
3.5-day-old	20	17.2 ± 2.0	1.00 ± 0.8
6.5-day-old	30	3.21 ± 1.3	6.40 ± 2.1
9.5-day-old	35	2.06 ± 1.0	15.5 ± 3.0

*Mean of five notochords ± standard deviation.

Table 2. [^{3}H]TdR labelling index and pyknotic index of aging notochords in vitro.

Incubation time (in vitro)	[^{3}H]TdR uptake/DNA content (cpm/μg)*	[^{3}H]TdR labelling index (%)*	Notochord cells with pyknotic nuclei (%)*
2 h	472 ± 116	58.1 ± 12	1.95 ± 1.4
3 days	292 ± 70	22.1 ± 2.7	11.5 ± 2.1
6 days	160 ± 60	12.7 ± 2.7	22.7 ± 1.7

*Mean of five notochords ± standard deviation.

Cell death evaluation

Measuring the number of dying cells in aging notochords in the different groups in vivo and in vitro is an essential step to study the mechanism of age-dependent involution in the notochord. Using pyknosis as a morphological parameter for cell death, the number of notochord cells with pyknotic nuclei was counted. The measurements were done on different sections of five notochords at the three stages of development.

Results

The simultaneous observation of proliferation and cell death might help to answer the question of whether involution can be attributed to a programmed intrinsic process or is influenced by the microenvironment in vivo.

DNA synthesis

In vivo. Autoradiographic results for the different stages examined demonstrated that the [^{3}H]TdR-labelled notochord nuclei were concentrated mainly at the periphery of the notochords (fig. 1A). There was a stage-dependent decrease in [^{3}H]TdR uptake by notochord cells (fig. 1A, 1B). The 3.5-day-old notochords gave a high [^{3}H]TdR labelling index which decreased sharply in the 6.5-day-old notochords. The labelling index decreased more gradually in the 9.5-day-old notochords (table 1).

In vitro. Liquid scintillation counting showed [^{3}H]TdR uptake by the 3.5-day-old notochords was high and

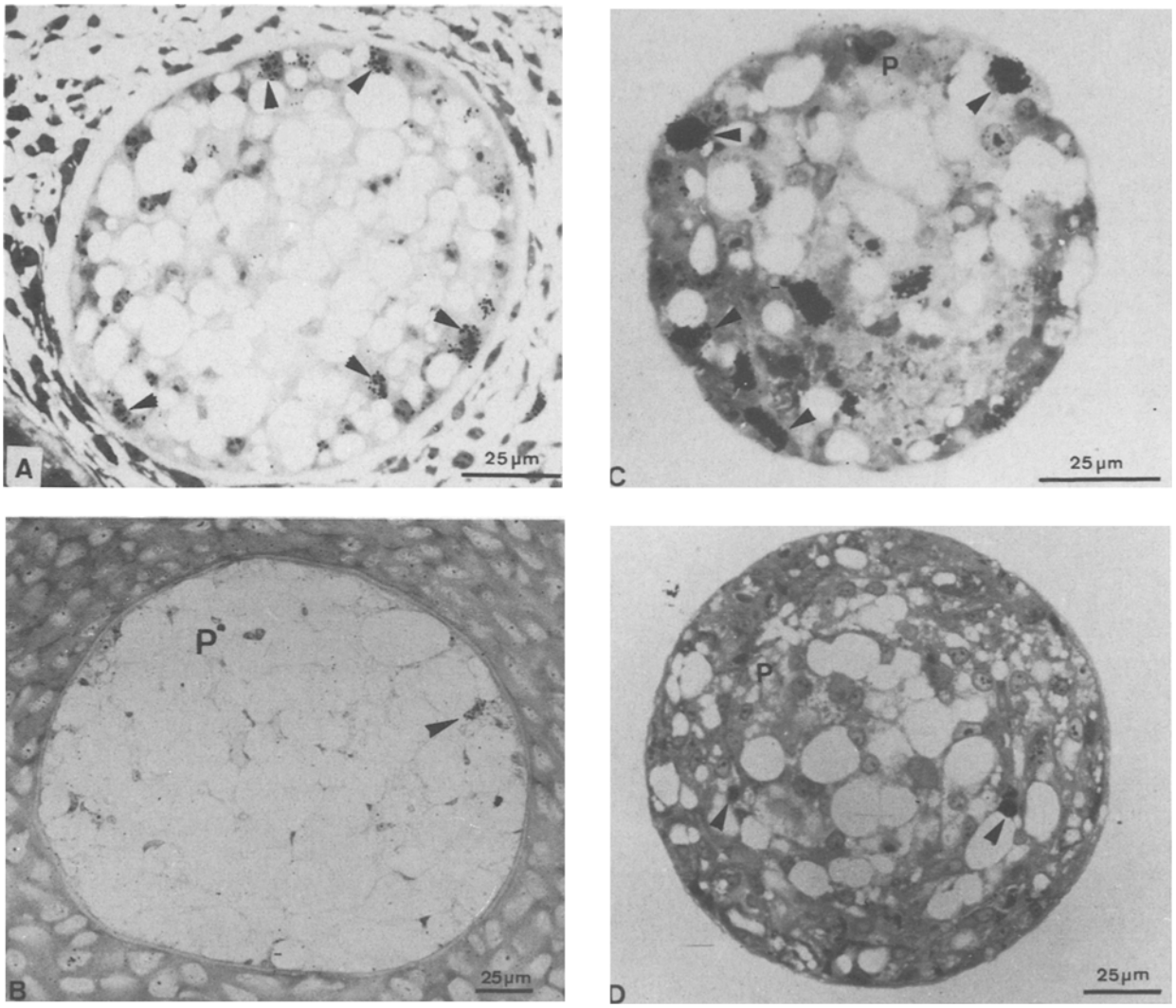

Figure 1. Histoautoradiographs showing nuclei of notochord cells of chick embryos, which were labelled with [^{3}H]thymidine (arrowhead), in vivo (A, B), (C, D) in vitro. (*A*) 3.5-day-old notochord. (*B*) 9.5-day-old notochord. (*C*) 3.5-day-old notochord after 2 h in vitro. (*D*) 3.5-day-old notochord after 6 days of in vitro incubation. P = pyknotic nucleus. Bar: 25 µm.

markedly decreased after 3 days of in vitro incubation. The lowest [^{3}H]TdR uptake by notochord cells (table 2) is demonstrated after 6 days in vitro. In autoradiographic results, the labelled cells were distributed in the notochord at random (fig. 1C, 1D). Some notochord cells were more intensively labelled than others in the same section. The [^{3}H]TdR labelling index showed an age- dependent reduction in notochords in vitro (table 2). For the selected age groups, the [^{3}H]TdR labelling index of the notochord cells was higher in vitro than in vivo. The relative reduction of the labelling index in aging notochords was more pronounced in vivo than in vitro (tables 1 and 2).

Cell death

In the different age groups, the notochord cells with pyknotic nuclei in vivo and in vitro are distributed at random (fig. 1). Pyknosis is not frequent in 3.5-day-old notochords in vivo, but the number of cells with pyknotic nuclei increased about six-fold in 6.5-day-old notochords. In 9.5-day-old notochords, the pyknotic nuclei increased nearly three-fold more than in the 6.5-day-old age group (table 1). Apparently there is an age-related increase of pyknosis in vitro similar to that seen in vivo (fig. 1). However, in notochords after 3 days in culture, the number of pyknotic nuclei was about 10-fold higher than in notochords at the onset of incubation. After 6 days in vitro, the number of pyknotic nuclei in the notochords was two-fold higher than in cultured notochords at 3 days (table 2). To summarize, as embryonic age progressed, the number of pyknotic nuclei in notochords increased in vitro and in vivo, and this process is more pronounced in vitro (tables 1 and 2).

Discussion

As embryonic age progresses, the notochord, which is considered to be the transient embryonic axis of the developing spinal column, rapidly undergoes progressive degeneration. This age involution could be an intrinsic characteristic of the notochord or might be influenced by the in vivo microenvironment. To distinguish between both possibilities, mesenchyme-free notochords were cultured in vitro, preserving their organotypic morphology, and comparison of cell proliferation and cell death in vivo and in vitro was performed.

Our in vivo results are in agreement with those previously described in the literature and demonstrate an age-related reduction of [^{3}H]TdR incorporation by aging notochords. In our data, the [^{3}H]TdR incorporation can still be visualized in notochords at stage 35 and does not stop after stage 31 as previously demonstrated[8]. The difference may be related to the methodology used, e.g. the site of injection, or incubation time in the presence of [^{3}H]TdR. From our present results, the marked reduction in [^{3}H]TdR uptake by notochords at stage 30 versus stage 20 is not as sudden as that reported by Shapiro[8]. He demonstrated high [^{3}H]TdR uptake by notochords at stages 22 and 27, which markedly diminished at stage 31[8]. However, in our previous results, the quantitative estimation of [^{3}H]TdR incorporation by notochords at different developmental stages in the period between 3.5-day-old (stage 20) and 6.5-day-old (stage 30) notochords showed a rather gradual decline[2].

In our current in vitro results, the mesenchyme-free notochords showed a reduction in [^{3}H]TdR incorporation during aging similar to that seen in vivo. This indicates that the process of age-dependent involution in notochords might be due to an intrinsic programmed process rather than to an extrinsic factor, as suggested by some authors[6,9]. From our results, it is difficult to conclude that the difference in [^{3}H]TdR incorporation by aging notochords, in vivo and in vitro, can be attributed to the in vivo environment alone. However, the in vitro conditions may have an impact on the aging process in the notochords. There may be a stimulatory effect on the cycling fraction of notochord cells, visualized by the higher [^{3}H]TdR incorporation by notochord cells in vitro than in vivo. Alternatively, the progressive reduction in the incorporation by aging notochords in vitro could be related in part to spontaneous degeneration provoked by culturing. However, as the rate of reduction of [^{3}H]TdR incorporation by aging notochord cells in vitro is less pronounced than in vivo, this suggests a limited effect of the in vitro environment. Moreover, morphological examination of aging notochords in culture shows no signs of deterioration in our in vitro system[3].

At the level of light microscopy, pyknosis is considered an indication of cell death[7]. From our results, pyknosis was demonstrated in vivo at stage 20. During the aging process, the notochord cells with pyknotic nuclei showed an age-dependent increase. This is in agreement with the results described by Jurand concerning the frequency of pyknotic nuclei from stage 25[5]. In our in vitro results, the mesenchyme-free notochords show a similar age-dependent increase in pyknosis as found in vivo. This might support our concept that the process of age-dependent involution of the notochord is an intrinsic process. However, the number of pyknotic nuclei, which was higher in vitro than in vivo, suggests a high turnover of notochord cells in vitro. This might be connected with the fact that the [^{3}H]TdR labelling index of the notochord cells is higher in vitro than in vivo.

The observation that the distribution of pyknotic nuclei is random in the notochord means that the process of diffusion is homogeneous and the central region is not deficient in nutrient supply. This is not in agreement with the hypothesis that the notochord undergoes progressive cell degeneration due to lack of blood supply and thickening of the perinotochordal sheath[6].

We can conclude that the process of involution in aging notochords seems to be an intrinsic programmed process, which might be influenced by the microenvironment in vivo. However, the exact mechanism of this intrinsic scheduled process of involution is not yet clearly understood and will be the subject of our future study.

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