Cell Tissue Res (1990) 260:507–511

Cell and Tissue Research

Nerve growth factor in skeletal tissues of the embryonic chick

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Accepted November 29, 1989

Summary. This study demonstrates, via immunohistochemistry and bioassay, the presence of NGF in embryonic bone and cartilage of the chick. Embryos were killed on days 6–9 of incubation at 12 h intervals, and on days 10–18 at 24 h intervals. Paraffin-embedded sections of hind limbs or buds were immunostained with a polyclonal antibody against NGF and the biotin-avidin-horseradish peroxidase technique. Immunostaining was positive in both bone and cartilage, with cartilage staining more intensely. For bioassay, bones from the hind limbs of 9- and 12-day embryos were fast-frozen, lyophilized, and homogenized with Medium 199 (M199). Dorsal root ganglia from 8-day embryos were cultured for 24–36 h with rooster plasma, M199, and varying concentrations of bone homogenate. Significant neurite outgrowth was seen, with the greatest response elicited by 12-day bone homogenate. Addition of anti-NGF to the cultures abolished neurite outgrowth. The results indicate that NGF is present in cartilage and bone of the chick embryo; it may determine the density of sympathetic innervation to the developing skeletal tissues.

Key words: Nerve growth factor – Osteogenesis – Chick embryo

Nerve growth factor (NGF) is a polypeptide which is essential for the development and maintenance of the sympathetic nervous system (Levi-Montalcini and Angeletti 1968). It can direct the growth of axons along its concentration gradient (Gunderson and Barrett 1979). Although NGF has been detected in a variety of embryonic and adult tissues (Chamley et al. 1973; Ebendal et al. 1980), it has not yet been reported in bone or cartilage. Singh et al. (1982), Herskovits and Singh (1984), and Sandhu et al. (1987) observed that bone deprived of its sympathetic innervation showed reduced bone deposition and mineralization, and increased resorption; they postulated that sympathectomy may cause the loss of a trophic influence important in osteogenesis. NGF might be such a trophic factor. The present study utilized immunohistochemistry and bioassay to demonstrate the presence of NGF in bone and cartilage cells of the chick embryo.

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Materials and methods

Immunohistochemistry

Thirty-two fertile eggs (White Leghorn chicken, Hall Bros. Hatchery, Wallingford, Conn., USA) were incubated at 37° C and 60% humidity. Calculation of incubation age of embryos was begun 6 h after start of incubation; this allowed the eggs time to reach incubator temperature and begin development. From days six to nine of incubation, two chicks were harvested at 12-h intervals; from days 10 to 18 they were harvested every 24 h, for a total of 16 developmental age groups.

Hind-limb buds or hind limbs (depending on age) were fixed for 24-h in a solution of 95% ethanol and 1% glacial acetic acid (Jacobsen et al. 1983). Femora of chicks of embryonic age 13 days and older were dissected free of soft tissue. Five-μm, sagittal sections were prepared; during embedding, paraffin temperature was kept at 56° C for preservation of the antigen. Experimental sections were incubated sequentially (Hsu et al. 1981) with (1) rabbit polyclonal anti-NGF (Collaborative Research, Lexington, Mass., USA), (2) biotinylated rabbit IgG (Vector Laboratories, CA), and (3) avidin horseradish peroxidase complex (Vectastain ABC reagent, Vector Laboratories), for 30 min each, and with 0.02% hydrogen peroxide-diaminobenzidine tetrahydrochloride for five min. Controls were treated identically, but were not incubated with anti-NGF. Slides were examined at ×400. A brown precipitate of the peroxidase substrate indicated the presence of NGF. Intensity of staining was graded as heavy, moderate, or light.

Bioassay

At 9 and 12 days of incubation, bones of the hind limbs were dissected free of soft tissue and fast-frozen on dry ice. Ten limbs

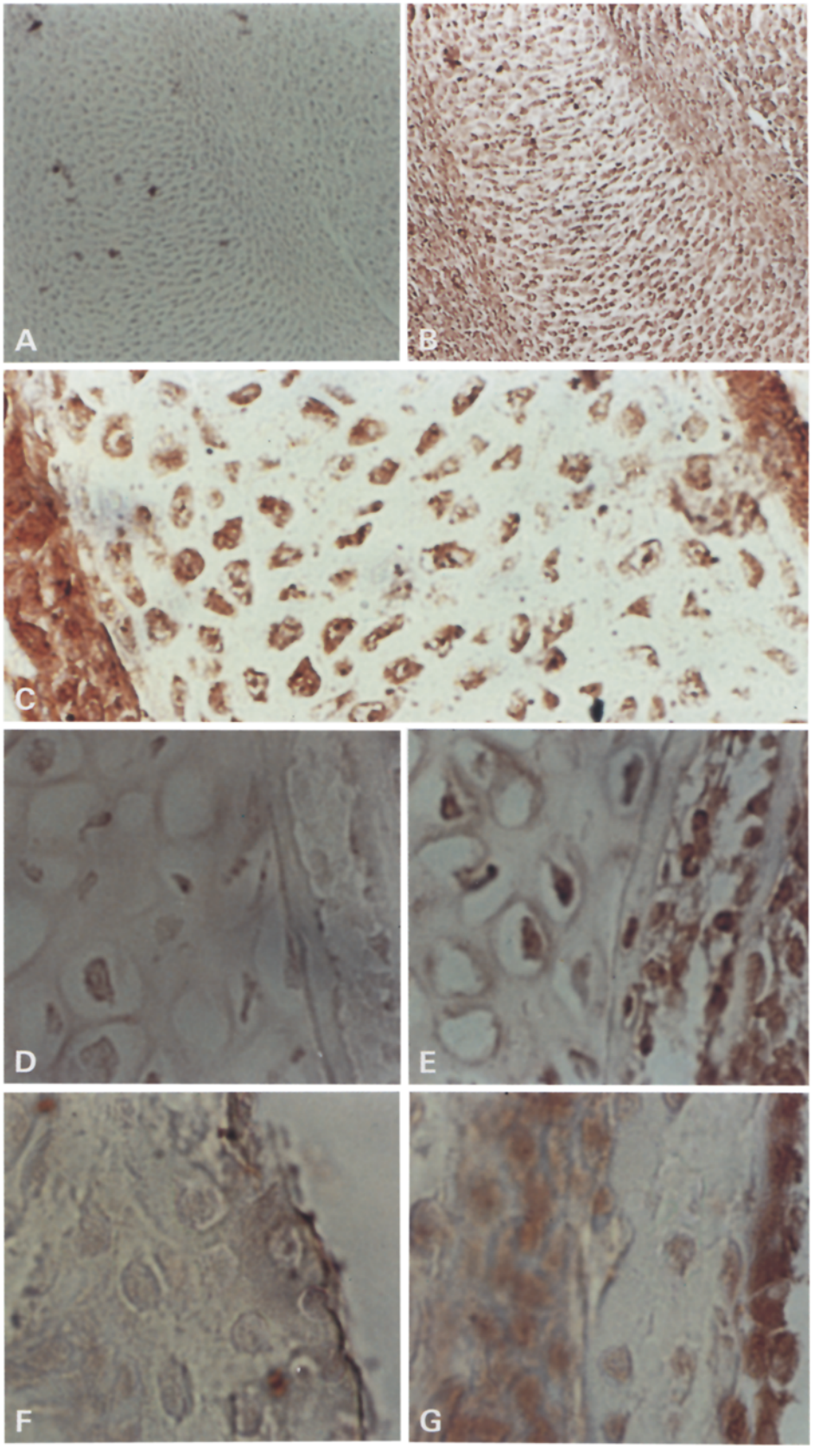

Fig. 1 A–G. Femora of chick embryos, immunostained for NGF.
A $6^1/_2$-Day control showing no stain, cellular detail difficult to discern;
B $6^1/_2$-day experimental, cells heavily stained a deep reddish brown;
C $7^1/_2$-day experimental, chondroblasts and chondrocytes stained, matrix unstained;
D neither cells nor matrix of 9-day control stained;
E 9-day experimental osteogenic cells and chondrocytes stained, matrix unstained;
F 14-day control limb, unstained;
G periosteal osteogenic cells, 14-day experimental, deeply stained; those surrounded by osteoid less so, i.e., closer to control in staining intensity. **A–B** $\times 200$; **C–G** $\times 600$

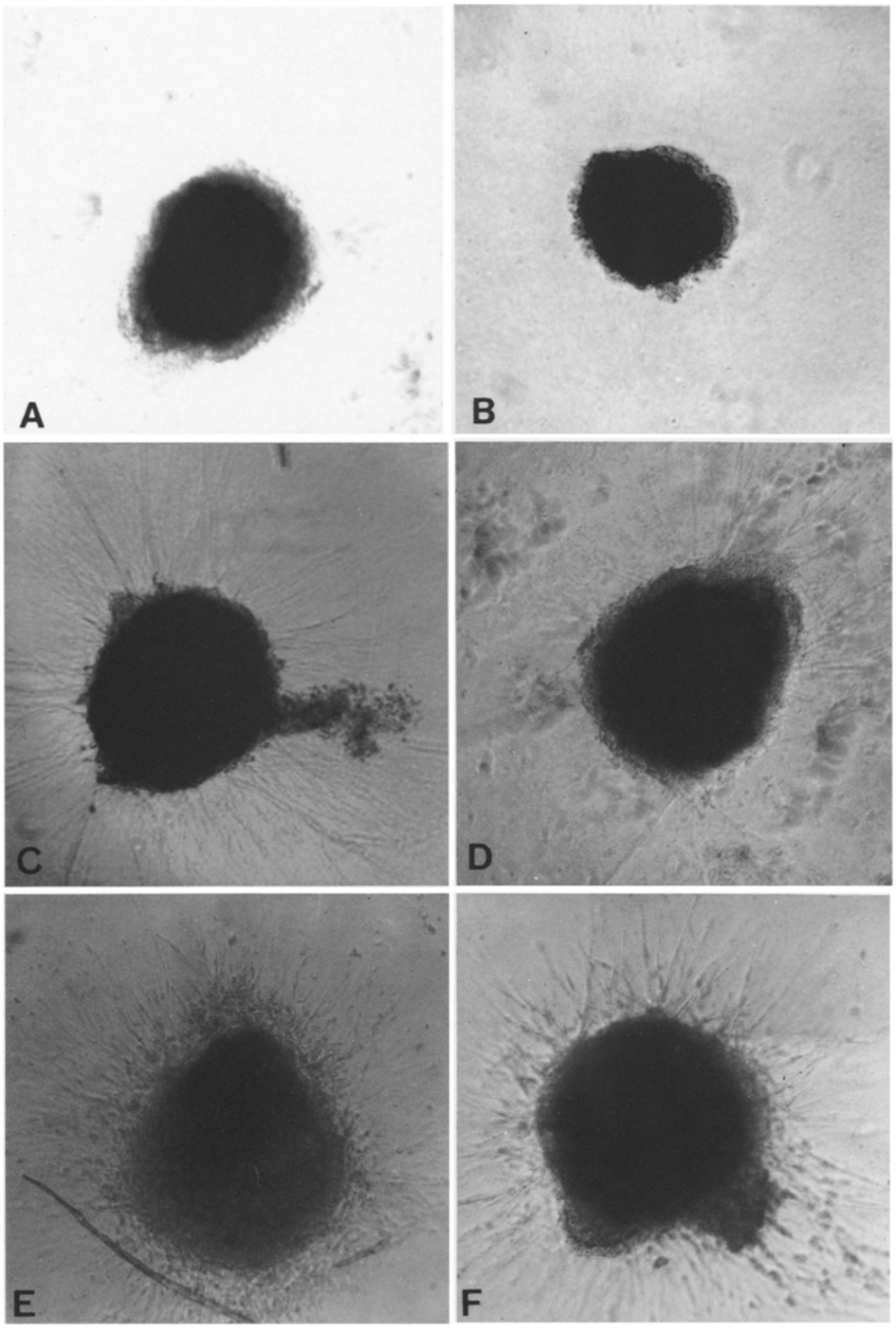

Fig. 2A–F. Dorsal root ganglia of 8-day chick embryos, incubated with M199, rooster plasma, and varying concentrations of bone homogenate.
A Negative control ganglion, incubated with rooster plasma and M199;
B negative control incubated with 10x dilution of 9-day homogenate and anti-NGF;
C positive control incubated with NGF;
D ganglion incubated with full strength homogenate of 9-day-embryo bone;
E incubation with 10x dilution of 9-day homogenate;
F incubation with 100x dilution of 12-day homogenate. ×25

from five embryos at each age were lyophilized, ground with a Corning manual tissue homogenizer, and Medium 199 (M199, a culture medium containing Earle's salts and L-glutamine; Gibco, Grand Island, NY., USA) was added in a volume/volume ratio of one part ground bone to nine parts M199. This suspension was designated the full strength concentration to be added to dorsal root ganglia in culture. In addition, 10x and 100x dilutions of the full-strength suspension were prepared. These three concentrations of hind limb homogenates (full strength, 10x, and 100x) were prepared from 9-day and 12-day-old embryos.

Ninety-six dorsal root ganglia, removed from 8-day-old chick embryos, were placed in culture dishes containing 20 µl of rooster plasma and 40 µl of M199. Three control culture media were prepared: the first, negative control, contained only plasma and M199; the second, positive control, contained 20 × µl of NGF (20 µg/ml, Collaborative Research), plasma, and M199; and the third control contained 20 µl of bone homogenate, 20 µl of anti-NGF, plasma, and M199. Experimental cultures contained 20 µl of bone homogenate, plasma, and M199. One ganglion per dish was suspended in the medium. Dishes were sealed with paraffin, and incubated at 37° C for 24–36 h. Neurite outgrowth from the ganglia was recorded as 0 (no growth) to 4+ (maximal growth), as described by Cohen et al. (1954).

Results

Immunohistochemistry

At the earliest developmental stages in the chick embryos examined (days 6, $6^1/_2$, and 7), cartilage and pre-cartilaginous condensations of mesenchyme in limb buds and hind limbs of experimental animals were intensely stained with immunolabel, having a deep reddish brown appearance (Fig. 1B). Control specimens showed no labeling, and were a pale beige, making tissue detail difficult to discern (Fig. 1A). By day $7^1/_2$, the cartilage template of the limb was well formed; and while chondrocytes and chondroblasts of the perichondrium did stain, the matrix was unstained (Fig. 1C). At day 9, osteogenic cells and newly formed osteoid were visible at the margin of the cartilage. The osteogenic cells, but not the osteoid, of experimental limbs were heavily stained (Fig. 1D, E). By day 11, the staining of chondrocytes was lighter than that of the osteogenic cells. At day 13 staining was light and many chondrocytic lacunae appeared empty. Small spicules of bone were visible from day 14 through 18. At day 14, osteogenic cells of the periosteal collar were still heavily stained; those surrounded by osteoid were more lightly stained, and were closer to the controls in their staining intensity (Fig. 1F, G). Staining intensity of all cell types dimished gradually; by days 17–18, there was little difference between experimental and control specimens.

Bioassay

The negative control cultures containing rooster plasma and M199 (Fig. 2A) showed no significant neurite outgrowth. Ganglia cultured with homogenate and anti-NGF (Fig. 2B) displayed no growth response whatsoever. Positive controls to which NGF was added had 4+ outgrowth (Fig. 2C).

Cultures with full strength concentrations of both the 9- and 12-day bone homogenates contained some debris from the undiluted homogenate; yet, neurite outgrowth from the ganglia was seen. The 9-day homogenate showed outgrowth in a range of 1+ to 2+, with a mode of 1+. The response to the 12-day homogenate was in the same range, but the mode was 2+ (Fig. 2D). Neurite outgrowth was more pronounced with the 10x dilution than with the full-strength bone homogenate (Fig. 2E); the range of outgrowth was 2+ to 3+, with a mode of 2+, for both the 9- and 12-day dilutions. Similar outgrowth was elicited by the still weaker 100x dilution and ranged from 2+ to 3+, with a mode of 2+ (Fig. 2F).

In sum, both the 10x and the 100x dilutions caused a more dense outgrowth of neurites than the undiluted homogenate. Neurites were also longer in the cultures containing diluted homogenates. While the experimental ganglia did not achieve the 4+ level of outgrowth of the controls treated with NGF, they did exhibit a positive growth response to the bone homogenates. Anti-NGF added to the cultures abolished the effect of the homogenates.

Discussion

These findings constitute the first demonstration of the presence of NGF in bone and cartilage of the chick embryo and extend earlier reports of its presence in several types of non-neuronal tissues (Chamley et al. 1973; Harper et al. 1979; Ebendal et al. 1980).

All cell types examined (mesenchymal cells, chondroblasts, chondrocytes, and osteogenic cells) stained positively, whereas matrix and osteoid did not. These histochemical findings were supported by the results of the ganglion-bioassay experiment, which is the classic test for the presence of NGF (Levi-Montalcini et al. 1954). Neurite outgrowth from dorsal root ganglia was elicited by culturing ganglia in homogenates of hind limbs or their buds. The homogenates used were mostly bone but also contained some cartilage. The most dilute homogenate used elicited the greatest level of outgrowth; this is consistent with unpublished observations that too high a concentration of NGF may be inhibitory (E.D. Bueker, personal communication).

In bioassay experiments, maximal neurite outgrowth was obtained with the 100x dilution of 12-day-embryo bone homogenate. Histochemical results reported here show that at that embryonic age, staining intensity in the cartilage cells of the femur had diminished, while bone cells were heavily stained. With increasing age, staining of bone cells became less intense. This may be due to a decrease in NGF receptor sites; Herrup and Shooter (1975) reported a sixfold drop in the number of these sites between embryonic days 14 and 16.

Intensity of immunohistochemical staining was dependent upon the developmental stage of the embryo, with limbs from younger specimens staining more intensely. Perhaps once the nerve supply is in place in the developing limb, the need for NGF diminishes. Innervation begins to reach the limb bud before the mesenchyme has differentiated into skeletal and muscle tissues in both the chick (Bennett et al. 1980) and the frog (Pollack and Kollros 1975). It is possible, therefore, that NGF in mesenchymal precursors as well as in differentiated tissues of the limb bud and limb may function as a chemotactic agent attracting innervation to the limb.

Our findings that the cells, but neither the cartilage matrix nor osteoid stain positively for the presence of NGF, correspond with the suggestions that directional growth of axons into the limb bud could best be explained by mesenchymal-cell synthesis of a growth factor (Bennett et al. 1980), and of substances that can govern directional growth of axons (Liu et al. 1979). The loss of such cell-derived factors may explain the reported decrease in osteogenic activity and increase in bone resorption following sympathectomy (Singh et al. 1982; Herskovits and Singh, 1984; Sandhu et al. 1987).

Hohmann et al. (1986) showed that sympathetic nerves in the periosteum release a peptide which stimulates bone resorption, thus providing further evidence of the relationship between the sympathetic nervous system and the development and maintenance of bone. It is possible that sympathectomy results in the loss of NGF, which would normally exert a trophic effect on

bone cells as suggested by Singh et al. (1981, 1982). The presence of NGF in embryonic bone and cartilage raises the possibility that during limb development NGF determines the degree of sympathetic innervation of the developing skeletal tissues.

Acknowledgements. This research was supported in part by BRSG grant RR 05332 and by the Anatomy Department Research Fund.

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