Early Changes in Parathyroid Hormone Response and Proteoglycan Synthesis of Chick Embryonic Femur Produced by Exposure to 6-Aminonicotinamide *in ovo*

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Abstract

The mechanism of induction of micromelia in 6-day-old chick embryo by 6-aminonicotinamide (6-AN) was investigated. Six-day-old chick embryo exposed to 6-AN did not show micromelia when tenfold excess of nicotinamide over 6-AN was co-administered. The ability of nicotinamide to prevent the induction of micromelia was partially offset after 4 hr of exposure to 6-AN and completely disappeared after 6 hr. The length of time necessary for the induction of micromelia was not affected by the concentration of 6-AN. These results indicate that exposure to 6-AN for only a short period of 6 hr is sufficient to commit the limb to micromelia and that cellular components involved in the induction of micromelia alter during this period. During this period, newly synthesized proteoglycan monomers typical of cartilage decreased in average molecular size, and isolated femora did not respond to parathyroid hormone (PTH) but to dibutyryl cyclic AMP to stimulate growth of cartilage in organ culture.

A nicotinamide analogue, 6-aminonicotinamide (6-AN) is known to affect limb development preferentially, thereby inducing micromelia (Landauer, W., 1957; Caplan, A. I., 1971; Caplan, A. I., 1972a, b; Seegmiller, R. E., *et al.*, 1972; Schubert, D., and Lacorbiere, M., 1976; Seegmiller, R. E., 1977; McLachlan, J. C., 1980). Although the precise mechanism has not yet been elucidated, it has been reported that 6-AN acts by suppressing NAD(P)-dependent reactions (Landauer, W., 1957; Dietrich, L. S., *et al.*, 1958; Verrusio, A. C., *et al.*, 1968; Ritter, E. J., *et al.*, 1975; Sheffields, V. C., and

Seegmiller, R. E., 1980) and sulfate utilization to form sulfated proteoglycans (Caplan, A. I., 1972a, b; Seegmiller, R. E., *et al.*, 1972; Overman, D. O., *et al.*, 1972; Honda, A., *et al.*, 1982), and by changing the developmental pathways of mesodermal cells (Caplan, A. I., 1972a, b; Schubert, D., and Lacorbiere, M., 1976)

As a first step toward elucidating the mechanism, it is important to determine the exposure time of chick embryo to 6-AN required to induce micromelia. The effect of 6-AN was completely offset by the co-administration of nicotinamide (Landauer, W., 1957; Caplan, A. I., 1971; Caplan, A. I., 1972a). To determine the shortest exposure

time required to induce micromelia with 6-AN, we administered nicotinamide to chick embryos at varying times after exposure to 6-AN. We found that no alleviating effect was observed when nicotinamide was introduced any time after 6 hr of exposure to 6-AN, indicating that at least 6 hr of exposure to 6-AN was sufficient to commit limbs to micromelia. During this short period, 6-AN causes cellular alterations that are resistant to the administration of nicotinamide.

To examine the cellular alteration which occurred during this period, it may be instructive to consider alterations in hormone actions which affect growth of cartilage and synthesis of cartilage-specific macromolecules. We showed previously that PTH stimulated the growth of embryonic chick limbs in organ culture (Kawashima, K., *et al.*, 1980a, b) but not the growth of micromelic limbs exposed to 6-AN for 5 days *in ovo* (Kawashima, K., *et al.*, 1982). In this paper, we examined whether exposure to 6-AN for 6 hr *in ovo* leads to a change in the response of limbs to PTH *in vitro*. We provide evidence that on short exposure (6 hr), the limbs lose their response to PTH. Moreover, we show that the average molecular size of newly synthesized proteoglycan monomers characteristic of cartilage decreases during this short term treatment.

Experimental Procedures

Materials

6-Aminonicotinamide and dibutyl cyclic AMP were purchased from Sigma Chemical Co. Nicotinamide was obtained from Wako Pure Chemical Industries Ltd., Japan. Parathyroid Hormone (Bovine, 1-34) was purchased from Beckman Instruments Inc.

Treatment of agents *in ovo*

A window was made in White Leghorn eggs after incubation at 37°C and 60% relative humidity for 6 days (stage 28-29) (Hamberger, V., and Hamilton, H. L., 1951) and various concen-

trations of 6-AN or nicotinamide in 50 μ l of Mg^{2+} , Ca^{2+} -free phosphate buffered saline (PBS(-)) were introduced. The same volume of PBS(-) was introduced into control eggs.

Isolation and cultivation of femur

Isolation of femora from chick embryos was performed as described previously (Kawashima, K., *et al.*, 1980a, b). The isolated femora were cultivated in a roller tube (Endo, H., 1960) in a chemically defined medium BGJb-HW2 (Endo, H., 1974). Pair-mate cultures were employed: one femur from the embryo served as a control and the other was cultivated in medium with PTH or dibutyl cyclic AMP.

Analysis of proteoglycan

Extraction and characterization of proteoglycans were carried out essentially as described by Hascall and Stanley (Hascall, V. C., and Stanley, W. S., 1969). ^{35}S -Labeled proteoglycans were extracted from isolated femora at 4°C in 4 M guanidine-HCl, 50 mM sodium acetate (pH 5.8) containing 1 mM phenylmethylsulfonylfluoride for 24 hr. Solid CsCl was added to the extracts to give a density of 1.50 g/ml and a density gradient was formed by sedimentation at 40,000 r.p.m. for 44 hr at 22°C. The bottom fractions ($\rho=1.53$) were pooled. Samples were dialyzed against the extraction solution and condensed by Sephadex G-100. The proteoglycan solution in 4 M guanidine-HCl containing 50 mM sodium acetate (pH 5.8) was layered on a linear 5-20% sucrose gradient in 4 M guanidine-HCl containing 50 mM sodium acetate (pH 5.8). The sedimentation was carried out at 38,000 r.p.m. for 14 hr at 4°C (Kimata, *et al.*, 1974).

Results

Effect of nicotinamide on induction of micromelia by 6-AN

To confirm the existence of the counteraction of nicotinamide to the teratogenic action of 6-AN, we examined the dose-dependency of the effect on the length of embryonic chick femora.

As shown in Fig. 1, the length resumed exponentially with the increase in the concentration of nicotinamide up to 50 μ g/egg.

Higher concentrations than 100 $\mu\text{g}/\text{egg}$ resulted in complete resumption. Accordingly, a tenfold excess of nicotinamide over 6-AN was used in subsequent experiments.

Time necessary for induction of micromelia by 6-AN

If the effect of 6-AN is completed in a specific period, the administration of nicotin-

Fig. 1. Dose-dependent effect of co-administration of nicotinamide and 6-AN on induction of micromelia. The concentrations of nicotinamide indicated were administered with 6-AN (10 $\mu\text{g}/\text{egg}$) to 6-day-old chick embryos. The eggs were incubated for 3 day and then the femora were isolated and their length was measured. Length were shown as percentages for the length of control femora ($5.90 \pm 0.24\text{mm}$). Points are means for five samples and standard deviation was so small that its representation did not exceed the diameter of each point.

Fig. 2. Effect of administration of nicotinamide after exposure to 6-AN on induction of micromelia. Varying concentrations of 6-AN (5 ($\blacktriangle$), 10 ($\bullet$), 20 ($\blacksquare$) $\mu\text{g}/\text{egg}$) were administered to 6-day-old chick embryos. Nicotinamide (100 $\mu\text{g}/\text{egg}$) was administered at the indicated times after exposure to 6-AN. After further incubation of the embryos for 3 days, the femora were isolated and their length was measured. Values are shown as percentages of the length of control femora (6.03 ± 0.29). The length of femora of embryos that were not treated with nicotinamide is shown by open symbols. Points are means for five samples and standard deviation was so small that its representation is buried in each symbol.

amide after this period should not modify the effect of 6-AN at all.

Nicotinamide alleviated the effect of 6-AN when it was administered within 2 hr after exposure to 6-AN (Fig. 2). However, its effect in preventing micromelia gradually decreased thereafter, and it had no effect after exposure to 6-AN for 6 hr or more. We also examined the relationship between the concentration of 6-AN and the time necessary for the induction of micromelia. The results showed that the length of exposure required to induce micromelia was identical irrespective of the concentration of 6-AN used (5, 10 and 20 $\mu\text{g/egg}$) (Fig. 2).

Effect of 6-AN on proteoglycan population

Fig. 3 shows sedimentation profiles in sucrose gradient of sulfated proteoglycans synthesized by femora exposed to 6-AN for 6 hr as compared to those by control femora. The proteoglycan monomers synthesized by control femora were separated into two size classes on the gradient. The heavy proteoglycan (fraction 1–12) is synthesized specifically by chondrocytes and the light

proteoglycan (fraction 20–24) is synthesized by fibroblasts, blastodisc and spinal cord cells (Okayama, M., *et al.*, 1976). The figure shows that slowly migrated heavy proteoglycan monomers increased in 6-AN-exposed femora. The figure also shows that the overall proportion of heavy to light sulfated proteoglycans in 6-AN-exposed femora is similar to that in control femora.

Effect of 6-AN on the response of limbs to PTH

Embryos were treated *in ovo* in three different ways, as described in Fig. 4, and then the femora were isolated and cultivated in BGJb-HW2 containing PTH or dibutyryl cyclic AMP and measured their length. Both PTH and dibutyryl cyclic AMP stimulated growth of control femora (Fig. 4A). In contrast, PTH had no stimulatory effect on femora isolated from embryos that had been exposed continuously to 6-AN (Fig. 4B). The loss of response to PTH was also fixed in femora that had been exposed to 6-AN for 6 hr before administration of nicotinamide (Fig. 4C). In femora exposed to

Fig. 3. Sedimentation profiles in sucrose gradient of proteoglycans from control (●) and 6-AN (10 $\mu\text{g/egg}$) exposed (○) femora. Six-day-old chick embryos were labeled with (^{35}S) sulfate (10 $\mu\text{Ci/egg}$) for 2 hr at 37°C after exposure to 6-AN for 6 hr. ^{35}S -labeled proteoglycans were extracted from isolated femora and analyzed by sedimentation of sucrose gradient as described under Experimental Procedures. Fractions 1–12 and 20–24 were collected as heavy (H) and light (L) proteoglycans respectively.

6-AN with or without subsequent administration of nicotinamide (Fig. 4B and 4C), however, growth stimulation by dibutyryl cyclic AMP was observed as in the case of control femora (Fig. 4A).

Discussion

We have investigated the mechanism of induction of micromelia by 6-AN. This report focused on how long 6-AN had to act to induce micromelia and what early events are involved in the induction of micromelia.

Exposure to 6-AN for 6 hr was enough for the completion of micromelia and the length of exposure time to induce micromelia was not affected by the concentration of 6-AN (Fig. 2). These results indicate that changes in cellular components are necessary to induce micromelia. We showed previously (Kawashima, K., *et al.*, 1980a, b) that growth of isolated embryonic chick femora cultivated in a chemically defined medium BGJb-HW2 was stimulated by PTH or dibutyryl cyclic AMP. PTH response in stimulating the growth of limb, however, was completely disappeared by the long exposure (from day 4 to day 9) of 6-AN *in ovo* (Kawashima, K., *et al.*, 1982). In addition, proteoglycan synthesis was altered by long term exposure to 6-AN. Seegmiller,

Fig. 4. Effect of PTH and dibutyryl cyclic AMP on growth of femora treated in three different ways. (A) Femora were isolated from 7-day-old chick embryos (control). (B) Femora were isolated from 7-day-old chick embryos that had been exposed to 6-AN ($10 \mu\text{g}/\text{egg}$) for 30 hr. (C) Femora were isolated from 7-day-old chick embryos that had been exposed to 6-AN ($10 \mu\text{g}/\text{egg}$) for 6 hr and treated with nicotinamide ($100 \mu\text{g}/\text{egg}$) and then incubated for 24 hr. These isolated femora were cultivated in BGJb-HW2 with (●) or without (○) PTH ($5\text{U}/\text{ml}$) and with (▲) or without (△) 1mM dibutyryl cyclic AMP. The length of the femora was measured every day. Points are means for five replicate cultures. The standard error was so small that its representation did not exceed the diameter of each point.

et al. (Seegmiller, R. E., *et al.*, 1980) demonstrated that when cartilage epiphyses isolated from 11-day-old chick embryos were cultivated *in vitro* in the presence of 6-AN for 17 hrs, the molecular weight of chondroitin sulfate and the ratio of the amount of chondroitin-4-sulfate to 6-sulfate were altered. Honda *et al* demonstrated that three day's exposure of 6-AN to chick embryo *in ovo* (day 4 to day 7) resulted in the inhibition of total glycosaminoglycan synthesis and of transition of mesenchymal to cartilage specific proteoglycans with an increase in the ratio of low sulfated glycosaminoglycans (Honda, A., *et al.*, 1982). Exposure of 6-AN to 10-day-old chick embryonic limb for one day *in vitro* also impaired the glycosaminoglycan synthesis (Iwama, M., *et al.*, 1983). We therefore investigated whether PTH response and the structure of sulfated proteoglycans of the femora were altered by short time exposure (6 hr) to 6-AN *in ovo*. Fig. 4 shows that after even short exposure to 6-AN (6 hr) the limbs lose their response to PTH. Moreover, during this period, proteoglycan monomers typical of cartilage decreased in average molecular size (Fig. 3). As previously mentioned, PTH stimulated the synthesis of proteoglycan in chondrocytes in cell culture (Suzuki, F., *et al.*, 1976) and in chick embryonic limb in organ culture (Kawashima, K., *et al.*, 1980a, b). These observations, therefore, imply that exposure to 6-AN would primarily cause changes in the response of chondrocytes to PTH and secondarily changes in the metabolism of chondrocyte-specific proteoglycan. It is possible that the changes resulting in micromelia involve the absence of PTH dependent cartilage growth and differentiation. The altered extracellular macromolecules such as proteoglycan and collagen may be involved in the maintenance of micromelia.

Poly (ADP-ribose) is a variably sized homopolymer synthesized from NAD and modifies chromosomal protein. Cherney *et al.* (Cherney, B. W., *et al.*, 1985) showed

that poly (ADP-ribose) synthetase, which catalyzes the synthesis of poly (ADP-ribose), transiently increased when chick embryonic mesenchymal cells differentiate into muscle and cartilage. Therefore, modification of chromosomal proteins by poly (ADP-ribose) may be one of the important events in normal development of cartilage. We found the inhibition of poly (ADP-ribose) synthetase activity and the changes in *de novo* synthesis of chromosomal proteins in the femora exposed to 6-AN for 6 hr *in ovo* (data not shown). Since changes in *de novo* synthesis and modification of chromosomal proteins occur during an extensive reprogramming of genetic expression, it seems possible that exposure to 6-AN causes changes in the transcription in chondrocytes and results in loss of responsiveness to PTH, probably involving deterioration of PTH receptor. It is felt that suppression of gene expression of PTH receptor would be an early event in inducing micromelia.

Another possible mechanism inducing micromelia by 6-AN is disturbance of NAD(P)-dependent enzyme reaction. 6-AN markedly lowers the level of ATP in limb bud (Sheffield, V. C., and Seegmiller, R. E., 1980; Honda, A. *et al.*, 1982). These studies suggest that 6-AN forms an abnormal NAD analogue which inhibits the activity of NAD(P)-dependent enzymes associated with the production of ATP. 6-AN acts preferentially on limb to induce micromelia. However, it is not known whether this impairment of the NAD(P)-dependent enzyme reaction occurred specifically in limb or not. Comparative studies on limb and the other tissues are necessary. Chondrocytes, however, require a large amount of ATP for the formation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) which is essential for the synthesis of sulfated proteoglycans, a major component of cartilage matrix. The decrease in ATP content, therefore, may be more sensitive to inhibition of differentiation and proliferation of chondrocytes than of

other cells, thereby inducing micromelia.

Prostaglandin E_1 and E_2 also stimulate *in vitro* chondrogenic differentiation of mesodermal cell of embryonic chick limb bud by increasing of cAMP (Kosher, R. T., and Walker, K. H., 1983) and play an essential role in limb development (Gay, S. W., and Kosher, R. A., 1985; Chepenik, K. P., *et al.*, 1984). Therefore the effects of PGE_1 and PGE_2 on the growth and differentiation of limbs of micromelia are an interesting subject. Experiments on this are in progress.

The present studies should serve as a basis for more detailed investigations on not only the mechanism of induction of micromelia by 6-AN but also the biological significance of PTH and extracellular macromolecules in limb development and differentiation.

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