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Biphasic influence of dexamethasone exposure on embryonic vertebrate skeleton development

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ABSTRACT

Dexamethasone (Dex) has anti-inflammatory and immunomodulatory properties against many conditions. There is a potential teratogenic risk, however, for pregnant women receiving Dex treatment. It has been claimed that Dex exposure during pregnancy could affect osteogenesis in the developing embryo, which still remains highly controversial. In this study, we employed chick embryos to investigate the effects of Dex exposure on skeletal development using combined *in vivo* and *in vitro* approach. First, we demonstrated that Dex (10^{-8}–10^{-6} μmol/egg) exposure resulted in a shortening of the developing long bones of chick embryos, and it accelerated the deposition of calcium salts. Secondly, histological analysis of chick embryo phalanxes exhibited Dex exposure inhibited the proliferation of chondrocytes, increased apoptosis of chondrocytes and osteocytes, and led to atypical arranged hypertrophic chondrocytes. The expression of genes related to skeletogenesis was also analyzed by semi-quantitative RT-PCR. The expression of ALP, Col1a2 and Col2a1 was decreased in the Dex treated phalanxes. A detectable increase was observed in Runx-2 and Mmp-13 expression. We next examined how Dex affected the different stages of skeletogenesis *in vitro*. Utilizing limb bud mesenchyme micromass cultures, we determined that Dex exposure exerted no effect on apoptosis but impaired chondrogenic cell proliferation. Interestingly, low dose of Dex moderately prompted nodule formation as revealed by alcian blue staining, but higher doses of Dex significantly inhibited similar chondrogenic differentiation. Dex exposure did not induce apoptosis when the chondrogenic precursors were still at the mesenchymal stage, however, cell viability was suppressed when the mesenchyme differentiated into chondrocytes. Alizarin red staining revealed that the capacity to form mineralized bone nodules was correspondingly enhanced as Dex concentrations increased. The mRNA level of Sox-9 was slightly increased in mesenchymal cell mass treated by low concentration of Dex. Mmp-13 expression was obviously up-regulated by Dex in both mesenchymal cells and primary chondrocyte cultures. And Col10a1 expression was also increased by Dex exposure in chondrocyte. In summary, we have revealed that different concentrations of Dex exposure during early gestation could exert a biphasic effect on vertebrate skeletal development.

Introduction

Dexamethasone (Dex) is a synthetic corticosteroid that is frequently used to relieve inflammations, such as swelling and fever. It has also been employed to treat certain types of arthritis, severe allergies, asthma and even certain types of cancers. It is generally known that glucocorticoids (GCs) are teratogenic and can induce cleft palate. Therefore, the cellular and molecular mechanism associated with cleft palate development has been investigated using animal models (Pinsky and Digeorge, 1965; Azziz and Ladda, 1990). Encephalocele and meningocele have developed in pregnant rhesus macaques when exposed to triamcinolone acetonide or Dex during gestation, and minor cranial skeletal abnormalities have developed in some monkeys (Jerome and Hendrickx, 1988). Previous reports have found that Dex affects the murine embryonic palatal shelf fusion, which could be rescued by pre-treating the embryos with vitamin B12 *in vitro* (Natsume et al., 1986; Lu et al., 2008). Consequently, the use of Dex has aroused wide public concern about its potential teratogenic effect on human embryonic development (Meyer-Bahlburg et al., 2004). However, it is still controversial whether Dex exposure during pregnancy can cause abnormal chondrogenesis and osteogenesis.

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Vertebrate skeletons are formed by either intramembranous or endochondral ossification (Gilbert, 2000). In intramembranous ossification, the osteoblasts directly differentiate from the richly vascularized mesenchyme which condenses to form a nodule. This morphogenetic process is responsible for forming flat bones of the cranium, part of the mandible and the clavicles. In contrast, endochondral bone formation consists of two steps. During the first step, the mesenchymal cells condense and undergo chondrogenesis to form a hyaline cartilage model, which is shaped like the prospective bone. These cartilaginous anlagen are ossified and the bone marrow is formed to give rise to mature bones. Most long bones of the limb, the vertebrae, the ribs, and the pelvis are generated by endochondral ossification — based on pre-existing hyaline cartilage primordia of future adult bone. During chondrogenesis, the chondrocytes proliferate, increase in size and produce an extracellular matrix in a dynamic process modulated by a precise and complicated signaling network (Adams et al., 2007). Subsequently, bone growth along the longitudinal axis relies on chondrocyte proliferation, hypertrophy and production of an extracellular matrix during both embryonic and postnatal periods. Because chondrocyte proliferation and differentiation are so important, any interference in these two processes would lead to shortening of the bone length (Rossi et al., 2002). It is now known that a large number of signaling molecules are involved in the regulation of skeletogenesis and exert regulatory effects on different stages of endochondral bone development. Chondrocyte proliferation, osteoblast differentiation and hypertrophic differentiation are the result of antagonistic interactions among several signaling pathways. Signaling genes related to this process are Ihh, PTHrP, FGF, BMP and Wnt, all of which must be expressed in a precise spatiotemporal fashion for proper development and pattern formation (Kronenberg, 2003; Tamamura et al., 2005; Adams et al., 2007).

GCs, synthesized and released from the adrenal cortex, and regulated by the hypothalamic–pituitary–adrenal axis according to metabolic requirement, are known to exert many effects on physiological functions and can retard the growth of children (Loeb, 1976; Swartz and Dluhy, 1978). GCs are able to reduce the velocity of new bone formation by directly inhibiting the deposition of calcium salt and indirectly affecting the level of sex hormones, implying that GCs exposure will reduce estrogen levels and augment parathyroid hormone levels in the blood. In addition to hormone synthesis, the expressions of paracrine factors such as insulin-like growth factor (IGF-I), growth hormone (GH) and NFκβ are also influenced (Heck et al., 1997; Carbone et al., 2012). These hormones and bioactive molecules are closely associated with bone homeostasis *i.e.*, maintaining the balance between new bone formation by osteoblast and bone resorption by osteoclasts. Any disturbance of this balance can lead to bone dysplasia, especially when some factors inhibit new bone formation and stimulate bone resorption (Olney, 2009). However, endogenous GCs are indispensable for bone formation, osteoblast differentiation and the maintenance of bone structure during embryonic osteogenesis (Mushtaq et al., 2002). When Dex, the synthetic glucocorticoid-like compound, is exposed to mesenchymal progenitor cells *in vitro*, it induces the cells to differentiate into chondrocytes as an adjuvant (Pevsner-Fischer et al., 2007; Zhang et al., 2010). Despite all these, the effect of Dex application on embryonic osteogenesis is still confused by the multitude of conflicting information. In this study, we have made use of chick embryos combined with mesenchymal cell mass cultures and a primary chondrocyte culture *in vitro*, to investigate the effects of Dex exposure on skeletal development during morphogenesis.

Materials and methods

Embryo manipulation

Fertilized Leghorn eggs were obtained from the Avian Farm of the South China Agriculture University (Guangzhou, China) and incubated in a humidified incubator (Yiheng Instruments, Shanghai, China) at 38 °C until the chick embryos reached the desired developmental stage HH10 (Hamburger and Hamilton, 1992; Ma et al., 2012). Then, the embryos were exposed to different concentrations of Dex (Sigma, MO, USA) or 0.1% DMSO (Sigma, MO, USA). Briefly, 100 μL of DMSO or various concentrations of Dex (10^{-8}, 10^{-9}, or 10^{-10} M) were carefully injected into a small hole made in the air chamber of the eggs, to achieve the final dosages of Dex are 10^{-6}, 10^{-7} and 10^{-8} μmol/egg respectively. After treatment, the embryos were further incubated for 7.5 days or 15.5 days before being harvested for analysis.

Alcian blue/alizarin red staining of whole embryos and morphometry

To visualize the vertebrate skeleton, the 9-day (E9) chick embryos were stained with alcian blue and alizarin red dyes as previously described (Solloway et al., 1998). Briefly, the embryos were fixed in 95% ethanol for 3 days and then the skin and viscera were carefully removed and post-fixed for 1 week. Next, the embryos were stained in 0.1% alcian blue and alizarin red (Solarbio, Beijing, China) dyes in 70% ethanol for 1 week and then cleared in 25% glycerol/1% KOH for 3 days. Finally, the embryos were treated in a grade series of glycerol. To make every part of the limb bone more visible, we carefully dismembered the skeleton and photographed them using a stereomicroscope (Olympus MVX10, Japan). Both the full bone length and the length of alizarin red stained portion of each humerus, radius, femur and tibia were quantified and analyzed by Image Pro-Plus (IPP 5.0, Media Cybernetics).

Histological analysis of HE, ALP, TUNEL and immunofluorescence staining

Some E17 embryos treated with Dex were harvested and fixed in 4% paraformaldehyde (PFA). The phalanxes of the embryos were decalcified in a 10% EDTA solution in 1 mM PBS (pH 7.4) for 2 days at 4 °C and then embedded in paraffin. The samples were serially sectioned at 5 μm thickness on a microtome (Leica RM2126RT, Germany). Longitudinal sections of these bones were produced and further stained with hematoxylin and eosin using a standard protocol for histological observations. For the histochemical staining of alkaline phosphatase (ALP) activity, a modified Gomori staining was performed as previously described (Imre and Fekete, 1983). The extent of apoptosis in the bone tissues was detected by TUNEL analysis, using an *in situ* Cell Death Detection Kit (Roche, Switzerland). The staining was performed according to the manufacturer's protocol and adapted for bone section labeling. Immunofluorescence staining was performed on some sections of the phalanxes as previously described (Li et al., 2013), using a monoclonal primary antibody against phosphorylated histone 3 (pH3, 1:100; Cell Signaling Tech, Boston, USA) and treated with the Alexa Fluor 555 anti-mouse IgG (1:1000; Life Tech, USA) secondary antibody. The sections were counterstained with 4′-6-Diamidino-2-phenylindole (DAPI, 5 μg/mL; Life Tech, USA) to reveal the nuclei and finally photographed by an Olympus IX51 microscope.

Histomorphometry was performed on TUNEL and pH3 immunofluorescence sections of phalanx growth plates using IPP 5.0 software. Quantitative measurements of pH3 positive cells and apoptotic cells were carried out as previously described (Silvestrini et al., 2000) with some modification. At least three sections, including the cartilage growth plate and the bone trabeculae, were obtained from each phalanx specimen. A representative field at 400× was used to obtain the cell counting.

Micromass cell culture

Micromass cultures were produced from limb bud mesenchymal cells as previously described (Delise and Tuan, 2002). Briefly, limb buds were dissected from HH23 chick embryos and treated with trypsin (0.25%; Life Tech, USA), the ectoderm was removed and limb buds were