AFRICAN JOURNAL OF MEDICINE and medical sciences

VOLUME 31, NO. 4 DECEMBER, 2002

ISSN 1116—4077

Afr. J. Med. med. Sci. (2002) 31, 325-327

The expression of the nuclear oncogenes *c-myc* and *c-jun* in the groove of Ranvier of the rabbit growth plate

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Summary
An immunohistochemical technique has been used to localise nuclear oncoproteins in the *groove of Ranvier*. The densely packed cells deep in the *groove* were immunoreactive positive for the *myc* protein. Cells and matrix in the same location were immunoreactive positive for the *jun* protein. These findings confirm the presence of proliferating cells in the *groove*. Injury to these cells is probably responsible for growth disturbances associated with trauma.

Keywords : *Groove of Ranvier, growth plate, oncogene expression, immunohistochemistry.*

Résumé
Une technique immunohistochemical a été employée pour localiser les oncoproteins nucléaires dans *la cannelure de Ranvier*. Les cellules en masse emballées profondément dans *la cannelure* étaient positif immunoreactive pour la protéine de *myc*. Les cellules et la matrice dans le même emplacement étaient positif immunoreactive pour la protéine de *jun*. Ces résultats confirment la présence des cellules de prolifération dans *la cannelure*. Les dommages à ces cellules sont probablement responsables des perturbations de croissance liées au trauma.

Introduction

There are four key sequential stages in the induction of cell proliferation (Figure 1). First, a growth factor binds to receptors on the outer surface of the cell membrane. Second, signal-transducing proteins on the inner surface of the cell membrane become activated. Third, secondary messengers are generated and these transmit the transduced signals to the nucleus. Finally, nuclear regulatory factors are activated [1] which transcribe the DNA. Following this, proteins are synthesised and mitosis occurs.

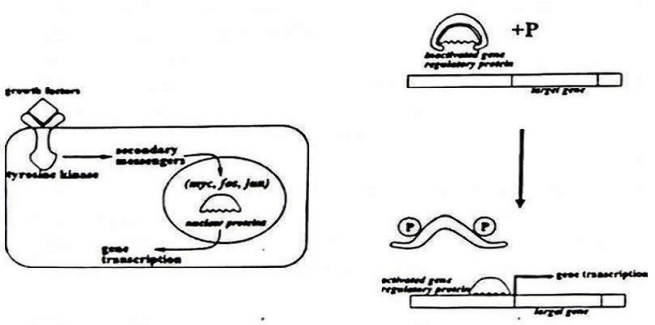

Fig. 1: A pathway for oncogene action.

Oncogenes [2] encode for the nuclear regulatory factors intimately involved in the proliferative response of cells [3,4]. The oncoproteins are synthesised in the cytosol and then transported into the nucleus where they bind specifically to DNA and thereby activate transcription. The *c-jun* and *c-myc* oncogenes apparently play a central role in musculo-skeletal differentiation [1,3-5]. In this pilot study, the expression of their protein products in the *groove of Ranvier* [6] has been investigated using an immunohistochemical technique.

Materials and methods

The growth plates of the hind limbs of a 5-day old and a 4-week old New Zealand white rabbits were studied (ie 4 femora and 4 tibiae). After removing the soft tissues, the specimens were fixed in fresh 10% buffered formalin for one week. They were then decalcified in Kristensen's solution (formic acid, sodium formate and distilled water) for a week and processed into paraffin wax. Longitudinal sections, 6u thick, were obtained and stained for the oncoproteins *myc* and *jun* using an immunohistochemical technique.

Immunohistochemistry:
The slides containing the sections were dewaxed in xylene and rehydrated through graded alcohols to water. Then they were washed with distilled water and modified TRIS.HCl buffered saline (TBS), pH 7.6, and then incubated with 1:20 normal rabbit serum (Dako-x0902) for 10 minutes to reduce background staining. Next, the sections were incubated with diluted primary antibody (mouse anti-*myc* 1:50, mouse anti-*jun* 1:20) (Novocastra, Newcastle) at room temperature for 1 hour. They were then washed in modified TBS, incubated with secondary antibody, biotinylated Rab a mouse Ig (Dako-E0413) in a dilution of 1:400, for 30 minutes and washed again. Avidin conjugated AP (Dako-D0365) in a dilution of 1:400 was added for 30 minutes thereafter the slides were washed with TBS and developed with a cocktail containing Levamisole 24mg, Fast Red TR 50mg, VAB pH 9.2 100ml and Naphthol as B_1PO_4 50mg. After washing, the slides were exposed to haematoxylin for 30 seconds, rinsed till blue in tap water and then mounted in aqueous mountant.

There were two controls: staining with primary antibody alone and staining with secondary antibody alone.

TBS was used as washing buffer between each stage of the staining and TBS/1% bovine serum albumin was used to dilute the antibodies.

Results

The *Groove of Ranvier* was very clearly demonstrated in all sections (Figure 2). A thin layer of new bone trabeculae, so-called bone bark, was directly applied to the periphery of the cartilaginous physis. A layer of densely packed plumpish cells with large nuclei was sandwiched between the bone bark and an outer fibrous sheath. The bone bark was observed to be continuous with the diaphyseal bone while the cellular and fibrous layers were in continuity with the layers of the periosteum of the diaphysis. The best reaction for *c-jun* was observed in the proximal and distal femoral epiphyses of 5-day old animals while the best reaction for *c-myc* was observed in the proximal femur of 4-week old animals. In general, c-*jun* immunoreactivity appeared to be intra- and extracellular while *c-myc* immunoreactivity was mainly intra-cellular.

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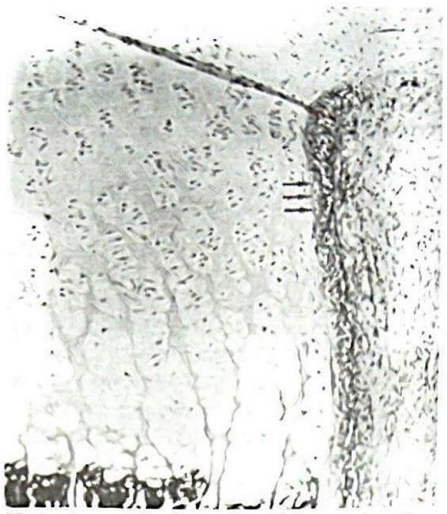

Fig. 2: Photomicrograph of a section of the proxima femur of a 5-day old rabbit stained for *c-jun* showing positive staining by the *Groove of Ranvier* (arrows); mag. x40

In the sections stained for *c-myc*, only the plumpish *groove* cells were positive staining. In higher magnifications, the layer of bone bark appeared featureless (Figure 3). It could be seen clearly to separate the cartilage cells of the physis from the *groove* cells. In the sections stained for *c-jun*, both the plumpish *groove* cells and matrix in their immediate vicinity were positive staining (Figure 4). Again, cells and matrix in the outer layer of the *groove* were not stained. The adjoining cartilage cells of the physis were also not stained in all cases.

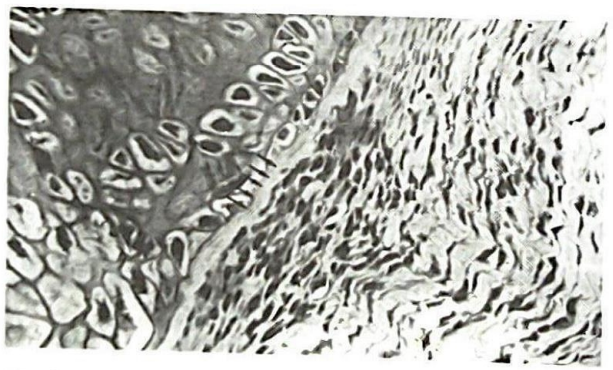

Fig. 3: Photomicrograph of a section of the proximal femur of a 4-week old rabbit stained for *c-myc*. Positive staining *groove cells*, to the right, are separated from the chondrocytes of the physis by a thin layer of amorphous material, the bone bark (arrows). mag x100

Fig. 4: Photomicrograph of a section of the proximal femur of a 5-day old rabbit stained for *c-jun* showing positive staining confined to the plumpish *groove* cells (middle stripm arrows); mag. x100

Discussion

According to these findings, *groove* cells express *c-myc* and *c-jun* oncogenes which raises the possibility that they are very rapidly proliferating and/or transforming cells.[7] previously described mitotic activity in this tissue. Rapid cell proliferation in the *groove* raises the possibility that the tissue has a biologically important function. The cells expressing oncogenes have previously been shown to express an osteoblastic phenotype [8]. There is evidence to suggest that they may be also cartilage-forming [9,10,11]. The expression of osteoblastic and chondrogenic phenotypes indicates a role in bone growth and Ogden [12] has claimed that the physis grows laterally through the mechanism of diametric expansion (Figure 5) analogous to 'ring formation' by trees. Traumatic damage to the *groove* has been associated with growth disturbances [8].

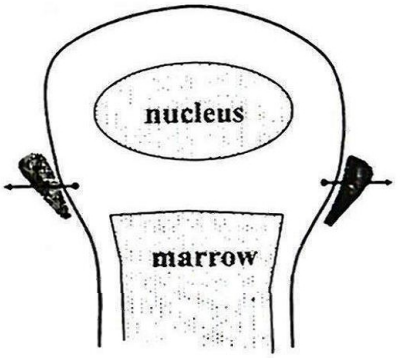

Fig. 5: Diagram: The 'diametric expansion' of physis (arrows) caused by groove cell growth (after Ogden 1979)

The origin of the oncogene-expressing cells of the *groove* is unclear [6] suggests the epiphyseal cartilage by a process of chondrolysis. There is supporting evidence. Peripheral defects of the physis appear to repair from within the cartilage in Ollier's disease and following x-ray induced injury of the rabbit growth plate [13,14]. Vital staining of the physis revealed stained cells 'migrating' towards the periphery of the physis [15]. Cartilage-associated type II collagen has been demonstrated in the *groove of Ranvier* [16]. This is all circumstantial evidence, however. [17] observed that when the physis was injured, all adjacent tissues – physis, *groove* and metaphysis - contributed to the regeneration. Type II collagen synthesis is not exclusive to chondrocytes; other cell types produce it [18]. Cartilage and bone cells have a common ancestry [19] and it is not surprising that bone cells may synthesise Type II collagen. The *groove* is separated from the cartilage of the physis by a bone bark (8, 20 and Figure 3). The *groove* and the physis develop independently in foetal and newborn calcaneus and talus [21].

The oncoproteins localised in this study are usually described as intracellular entities [1,3,4]. Exactly why the *c-jun* protein was also expressed in the extra-cellular matrix in these sections is not immediately apparent. Extra-cellular expression may be a reflection of quantity, cell membrane leakage, for example, consequent upon tissue preparation and/or additional function as an autocrine/paracrine. On the other hand, the procedure used to prepare tissue for immunohistochemistry may be responsible.

Conclusions

1. The expression of c-myc and c-jun oncogenes supports the proposition that there is active tissue proliferation in the *groove of Ranvier*.
2. The oncogene-expressing cells have previously been shown by others to be capable of expressing both osteoblastic and chondrogenic phenotypes which raises the possibility that they may have an important role in bone growth.

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