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Expression patterns of chondrocyte genes cloned by differential display in tibial dyschondroplasia

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Abstract

Tibial dyschondroplasia (TD) appears to involve a failure of the growth plate chondrocytes within growing long bones to differentiate fully to the hypertrophic stage, resulting in a mass of prehypertrophic chondrocytes which form the avascular TD lesion. Many biochemical and molecular markers of chondrocyte hypertrophy are absent from the lesion, or show reduced expression, but the cause of the disorder remains to be identified. As differentiation to the hypertrophic state is impaired in TD, we hypothesised that chondrocyte genes that are differentially expressed in the growth plate should show altered expression in TD. Using differential display, four genes, B-cadherin, EF2, HT7 and Ex-FABP were cloned from chondrocytes stimulated to differentiate to the hypertrophic stage in vitro, and their differential expression confirmed in vivo. Using semi-quantitative RT-PCR, the expression patterns of these genes were compared in chondrocytes from normal and TD growth plates. Surprisingly, none of these genes showed the pattern of expression that might be expected in TD lesion chondrocytes, and two of them, B-cadherin and Ex-FABP, were upregulated in the lesion. This indicates that the TD phenotype does not merely reflect the absence of hypertrophic marker genes, but may be influenced by more complex developmental mechanisms/defects than previously thought. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Growth plate chondrocyte; Gene expression; Differential display; Tibial dyschondroplasia

1. Introduction

Chondrocyte terminal differentiation in the growth plate is of pivotal importance in the process of endochondral ossification [1]. Chondrocytes from the resting zone of the growth plate proceed through a series of intermediate phenotypes: proliferating, prehypertrophic and hypertrophic, before reaching a terminally differentiated state [2,3]. Tibial dyschondroplasia (TD) is a common cause of bone deformity in broiler chickens which is characterised by an opaque avascular lesion in the metaphysis of long bones [4]. It occurs most frequently in the proximal tibiotarsus, although other joints may also be affected [5]. The lesion appears to contain predominantly prehypertrophic chondrocytes, with fully hypertrophic cells absent [6]. In addition, genes that are normally upregulated during chondrocyte hypertrophy tend to show a lower level of expression in the TD lesion. This has been shown to be the case for a number of genes, including matrix components [7–9], growth factors [10–12] and receptors [13]. These data support the conclusion that TD involves a failure of

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growth plate chondrocytes to reach the terminally differentiated hypertrophic state [6,14].

In order to test the hypothesis that TD is caused by the arrest of chondrocyte differentiation, we have used an in vitro model of chondrocyte differentiation [15,16], to isolate differentially expressed genes, and then compared the mRNA level of these genes in normal and TD lesion chondrocytes. If the above hypothesis is correct, then genes normally upregulated in hypertrophic chondrocytes should show reduced expression in the TD lesion. Here, we report the expression patterns of four differentially regulated genes cloned from chondrocytes by agarose gel differential display [17]. Surprisingly, two of these genes, ExFABP and B-cadherin, were upregulated in lesion chondrocytes, and were expressed at levels that were not compatible with the absence of hypertrophic chondrocytes from the TD lesion. Nor could this upregulation be explained by changes known to occur in lesion chondrocytes. This suggests that the TD phenotype may not merely reflect the absence of fully hypertrophic cells from the lesion [14,18], but may be due to more complex mechanisms than previously thought.

2. Materials and methods

2.1. Differential display

The identification of differentially expressed genes was performed by the technique of agarose gel differential display [19], modified to allow the use of total RNA [17]. Briefly, proliferating chondrocytes isolated from the growth plate of the proximal tibiotarsi of 3-week-old chickens were grown in culture for 6–7 days in the presence or absence of ascorbic acid, a substance known to induce differentiation to the hypertrophic state [15], and the cells harvested for the extraction of total RNA as described below. RNA was reverse transcribed using random hexamers and differential display was performed using a number of different combinations of two arbitrary 10-mer primers [19]. Bands that were reproducibly differentially expressed were excised from the gel and cloned into a TA vector (PCR-II; InVitrogen, the Netherlands). Differential expression was assessed by a non-radioactive dot blotting technique and semi-quantitative

RT-PCR, or by Northern blotting [17,20]. Four genes that were confirmed as differentially expressed in the chick growth plate, as well as in ascorbic acid-treated cells, were then assayed for altered expression in TD lesion chondrocytes (see below).

2.2. Chondrocyte isolation

Isolation and culture of growth plate chondrocytes for differential display was carried out as previously described [17]. To confirm the in vivo differential expression of genes isolated from chondrocytes grown in culture, separate sections were dissected from the top and bottom of normal growth plates, so as to represent cell populations of predominantly proliferating (P) and hypertrophic (H) chondrocytes. Material from these samples was diced into small cubes and chondrocytes isolated from their surrounding matrix by digestion with 0.3% collagenase A (Roche Diagnostics, UK) and 0.1% hyaluronidase (Sigma, UK) in Dulbecco's modified Eagle's medium (BRL Life Technologies, UK) for 4 h at 37°C. Undigested matter was removed with a 45-μm filter the cells washed with DMEM and pelleted for extraction of total RNA. Growth plate chondrocytes from TD and non-TD birds were obtained from broiler chicks (Ross strain) raised on a calcium/phosphorus imbalanced, TD-inducing diet, containing 7.5 g/kg calcium, 7.6 g/kg phosphorus and 25 mg/kg vitamin D_3, which resulted in an increased incidence of the disorder [7]. Chicks were sacrificed at 3 weeks of age, the proximal tibiotarsi split longitudinally and scored visually for TD according to a standard scale of severity [14]. Chondrocytes were isolated from the entire growth plate of TD and non-TD birds, with TD lesion material divided into small (S; TD score of 1 or 2) and large (L; TD score of 3 or greater) samples. Care was taken to include comparable areas of the growth plate for both TD and non-TD samples. Chondrocytes were isolated by collagenase digestion as described above and total RNA extracted directly from the pelleted cells.

2.3. RNA extraction

Total RNA was extracted from isolated chondrocytes using Ultraspec II according to the manufacturer's instructions (Biotecx, USA). Briefly, cells were

resuspended in 1 ml of Ultraspec II and the suspension passed repeatedly through an 18-gauge needle. After incubation on ice, the RNA was extracted with chloroform and the aqueous phase recovered. The RNA was then bound to RNATack resin (Biotecx) in the presence of isopropanol and recovered by brief centrifugation. After washing with 75% ethanol, the RNA was eluted in a small volume of 10 mM Tris (pH 8.0).

2.4. RT-PCR

Total RNA was reverse transcribed with random hexamers in aliquots of 1 μg using the Superscript preamplification system (BRL Life Technologies). PCR reactions were carried out in a volume of 10 μl in 200 μl thin-walled tubes using a Robocycler PCR machine (Stratagene, UK). The reactions contained TLA buffer [21,17], 0.05% W-1 detergent (BRL Life Technologies), 1 U of Taq polymerase (Advanced Biotechnologies, UK), in the presence of Taqstart antibody (Clontech, UK) and the equivalent of 10 ng of target RNA. The final concentration of the primers was 1 μM. The temperature profile of the PCR reactions was 95°C for 30 s, 58°C for 60 s and 72°C for 60 s. The number of PCR cycles was carefully titrated to ensure that the reactions were in the exponential phase. The number of cycles used were: B-cadherin 30 cycles, HT7 28 cycles, EF2 27 cycles, Ex-FABP 26 cycles and GAPDH 22 cycles. Ex-FABP was previously referred to as Ch21 [22]. Reaction products were run out on 3% NuSieve agarose gels (Flowgen, UK), stained with ethidium bromide and a record of the gel made using the Bio-Rad gel documentation system (Bio-Rad, UK). For comparison of TD and non-TD samples, band intensity was quantified using Molecular Analyst gel documentation software (Bio-Rad). Relative levels of expression were estimated in arbitrary units, with the control band assigned a value of 1. Each gene was assayed at least three times and the results collated and analysed statistically using a one sample *t*-test within the Statview statistics package (Abacus concepts, USA).

2.5. Gene specific primers

Ex-FABP: forward, 5′-GAC TTC TTC CTG

CGT GAG AAG G-3′; reverse, 5′-ATC AAC GCT GCA TTC CTC CTG G-3′. HT7: forward, 5′-CTG TGG GGA TGA CCT GAT CTC-3′; reverse, 5′-CTC TGA GCA TTA CCT GCT CTC-3′. B-cadherin: forward, 5′-GTG ATC CCT CCC ATT AAA GTT C-3′; reverse, 5′-CTC CTT CTC AAT GGT GAA GAT G-3′. EF-2: forward, 5′-TGA AGT TCA GCG TCA GCC CTG T-3′; reverse, 5′-TAG GAA TGC AAG CGT GGT CCT C. GAPDH: forward, 5′-TGT GAC TTC AAT GGT GAC AG-3′; reverse, 5′-ACA GAT CAG TTT CTA TCA GC-3′. The specificity of the primers was confirmed by cloning and sequencing of the PCR products (data not shown).

3. Results and discussion

3.1. Isolation of differentially expressed genes

In an initial attempt to isolate candidate genes for TD, we performed differential display [17] using total RNA isolated from TD lesion and normal growth plates. However, this approach was hampered by the lack of reproducibility of the banding patterns obtained with both RNA samples, and the number of false positives generated (D.J., unpublished). This was most likely due to the heterogeneity of the chondrocyte populations isolated. Due to the nature of the technique, differential display is highly sensitive to small variations in RNA abundance between samples, which renders comparison difficult [23]. To overcome this problem, we employed an alternative strategy that exploits the impaired differentiation of lesion cells. We used an in vitro model of chondrocyte differentiation [15,16] to isolate differentially expressed genes, and then compared the mRNA level of these genes in normal and TD lesion chondrocytes. If the hypothesis that TD is caused by the arrest of chondrocyte differentiation is correct, then genes normally upregulated in hypertrophic chondrocytes should show reduced expression in the TD lesion. Here, we report the expression patterns of four differentially regulated genes cloned from chondrocytes by agarose gel differential display [17] and show that their pattern of expression does not fit the above hypothesis.

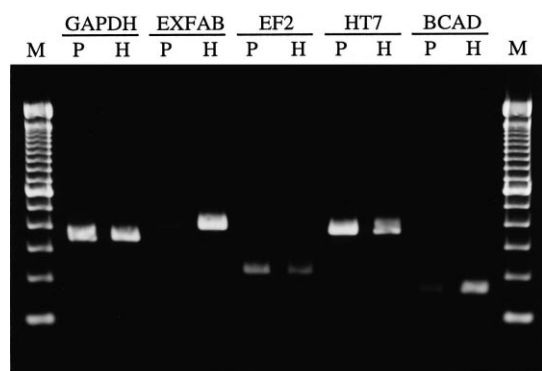In addition to the two gene fragments described

Fig. 1. Confirmation of differential gene expression in total RNA samples from populations of proliferating (P) and hypertrophic (H) growth plate chondrocytes. M = 100 base pair marker.

previously (EF2 and Ex-FABP [17]), a further two fragments were cloned by differential display and their differential expression in ascorbic acid treated chondrocytes confirmed (data not shown). These fragments were sequenced and shown to have homology to B-cadherin, a calcium-dependent cell adhesion molecule [24] and the cell surface antigen HT7, which is also involved in cell–cell interactions [25].

3.2. Confirmation of differential expression

As well as verifying the differential expression of these four genes in culture, their regulation in growth plate chondrocytes was also confirmed by comparing populations of predominantly hypertrophic and proliferating chondrocytes isolated from normal growth plates (Fig. 1). The results show that while HT7 and EF2 show moderate upregulation in proliferating chondrocytes, both Ex-FABP and B-cadherin are more highly expressed in hypertrophic cells.

The finding that expression of B-cadherin is upregulated in hypertrophic chondrocytes was somewhat surprising as there appears to be little cell–cell contact between chondrocytes of the hypertrophic zone, due to increased matrix production [26]. However, interactions between cadherins and matrix components such as integrins and collagens have been demonstrated [27–29]. Integrins have been shown to be important in regulating the progression of chondrocyte differentiation within the growth plate [16,30]. Therefore, a role for B-cadherin in cell–matrix interactions in the growth plate cannot be ruled out [31].

In addition, cadherins may be involved in apoptosis [31], which has been shown to occur with increased frequency in the hypertrophic zone [32,33].

HT7 is downregulated in hypertrophic chondrocytes from normal growth plates (Fig. 1). This may well be due to a reduction in cell contact in the hypertrophic zone of the growth plate, as HT7, like B-cadherin, is a cell surface glycoprotein involved in cell–cell interactions [25]. However, recent studies have shown that EMMPRINN, the human homologue of chick HT7, is closely associated with integrins on the cell surface, and that both these molecules

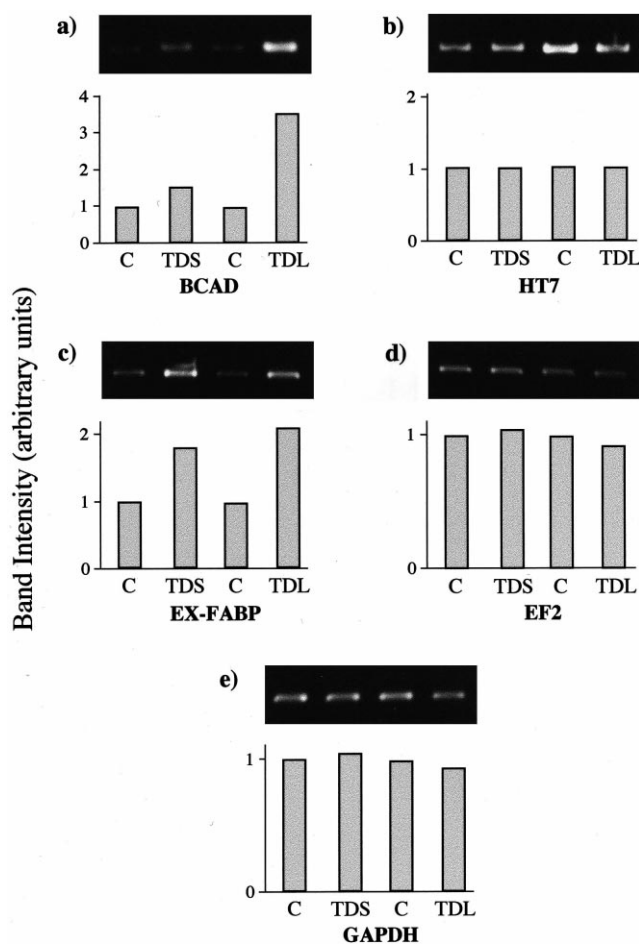Fig. 2. Expression of genes in total RNA samples from control and TD lesion chondrocytes. PCR products from normal control (C), small (TDS) and large (TDL) lesion samples, run out on agarose gels and stained with ethidium bromide. The level of gene product in lesion cells was expressed in relation to the band intensity in control cells run out in adjacent lanes on the same gel (arbitrary value of band intensity in control lane = 1).

may function to regulate matrix metalloproteinase activity [40]. The reduced expression of EF2, which is an elongation factor catalysing the ribosomal synthesis of polypeptides [41], is most likely a result of a lower level of metabolic activity in terminally differentiated hypertrophic cells [3].

The isolation of Ex-FABP from ascorbic acid treated cells was reassuring as this gene is a recognised marker of chondrocyte hypertrophy [46]. Ex-FABP, or extracellular-fatty acid binding protein, is a member of the lipocalin superfamily of proteins that bind and transport small hydrophobic molecules [47]. It is secreted into the extracellular matrix where it probably binds to long-chain unsaturated fatty acids, for which it has a high affinity [22].

3.3. Expression patterns in TD lesion chondrocytes

The expression patterns of these four genes were subsequently analysed in growth plate chondrocytes isolated from TD and non-TD birds using semi-quantitative RT-PCR. The GAPDH gene was used as a control to ensure that equal amounts of cDNA were compared between samples. Fig. 2 shows a typical result for each of the genes assayed in samples

from small and large TD lesions, together with a bar chart showing the relative level of expression in the TD and non-TD samples. The expression of EF2 and HT7 did not differ significantly between normal and lesion chondrocytes (Fig. 2 and Table 1). However, both B-cadherin (Fig. 2a) and Ex-FABP (Fig. 2c) were upregulated in chondrocytes from both small and large lesions. In addition, the trend in both cases was for increased differences in the larger lesions (Fig. 2).

The reason for the apparent upregulation of B-cadherin in lesion material is not clear. There is little evidence for increased cell–cell contact within the lesion and increased expression may simply be due to the particular phenotype of lesion chondrocytes. Whether B-cadherin is involved in apoptosis in the TD lesion is debatable. There are conflicting reports on the frequency of programmed cell death in lesion chondrocytes and both increased [34,35] and decreased [33] levels have been reported. One further possibility is that a low level of calcium in the lesion [36] is responsible for upregulation of B-cadherin. Although there are conflicting reports on the levels of calcium in dyschondroplasia [36,37], such a scenario agrees with published observations of the

Table 1
Statistical analysis of TD expression data for the four genes cloned by differential display

Gene	Sample[a]	n[b]	Mean	S.E.[c]	P[d]	Significance[e]
BCAD	S	7	0.167	0.053	0.02	$P < 0.05$
	L	5	0.488	0.119	0.01	$P < 0.01$
	S+L	12	0.301	0.073	0.002	$P < 0.01$
HT7	S	7	0.010	0.032	0.76	n.s.
	L	4	0.002	0.031	0.95	n.s.
	S+L	11	0.007	0.022	0.75	n.s.
Ex-FABP	S	6	0.252	0.040	0.003	$P < 0.01$
	L	4	0.315	0.054	0.01	$P < 0.01$
	S+L	10	0.277	0.032	< 0.0001	$P < 0.001$
EF2	S	6	0.008	0.042	0.86	n.s.
	L	4	0.039	0.023	0.19	n.s.
	S+L	10	0.110	0.027	0.69	n.s.
GAPDH	S	6	0.016	0.035	0.68	n.s.
	L	3	−0.034	0.055	0.60	n.s.
	S+L	9	−0.001	0.027	0.98	n.s.

The GAPDH gene was used as an internal control.
[a] S, small lesions; L, large lesions; S+L, small and large lesions combined.
[b] n = number of samples.
[c] S.E., standard error.
[d] P = probability value.
[e] Level of significance, n.s., not significant.

abundance and localisation of cadherins in osteoblasts cultured in low calcium medium [38]. Instead of remaining at the cell surface, cadherin staining was shifted to the cytoplasm and became much more intense in cells exposed to low calcium. This coincided with the appearance of a low molecular weight band in Western blots probed with a cadherin antibody. These observations are strikingly similar to our own recent results with protein lysates from TD lesions. Using a pan-cadherin antibody that recognises a wide variety of cadherins [39], at least two specific bands are visible on Western blots of both normal and TD growth plate chondrocyte lysates. However, one band of about 30 kDa, while barely detectable in normal growth plates, becomes extremely abundant in TD (DJ, unpublished observations). This is further evidence, albeit circumstantial, that the avascular microenvironment of the TD lesion can influence gene expression levels.

In lesion material, mRNA levels of HT7 and EF2 are the same as those in the normal growth plate, despite the absence of fully hypertrophic chondrocytes. This implies a reduction in the expression of both genes in the cells of the lesion, compared to prehypertrophic chondrocytes from normal growth plates. In the case of HT7, this may indicate a reduction in cell–cell contact in the lesion, or be related to a possible role in matrix metalloproteinase production, as described above. Interestingly, matrix metalloproteinase activity produced by lesion chondrocytes in vitro, has been shown to be reduced compared to normal growth plates [42]. For EF2, expression levels probably reflect a lower level of metabolic activity and/or protein turnover in lesion cells [43], as the cells are not proliferating or differentiating. Certainly, those enzyme activities which have been measured, such as alkaline phosphatase, lactate dehydrogenase carbonic anhydrase, esterase, triose phosphate isomerase and malate dehydrogenase, are lower in, or absent from, the lesion [14,44,45].

The reason for the increased expression of Ex-FABP in lesion cells is intriguing as upregulation of Ex-FABP is normally a marker of chondrocyte hypertrophy [46]. Interestingly, it is also homologous with p20k a protein often associated with quiescent cells [48]. It has been shown that proliferating cells are absent from the TD lesion [14]. Therefore, it is

possible that cell quiescence could account for the upregulation of Ex-FABP, or even that elevated levels of Ex-FABP prevent further cell division and terminal differentiation in the TD growth plate. Alternatively, the increased level of Ex-FABP mRNA may be due to the particular phenotype of TD lesion cells. In this context it is interesting to note that the upregulation of Ex-FABP has recently been confirmed at the protein level in chondrocytes and myoblasts treated with inflammatory agents in culture, as well as in chondrocytes from TD lesions and osteoarthritic cartilage [49]. It was suggested that increased expression of the protein in the TD lesion may be part of an acute phase response by lesion chondrocytes, although this has not yet been confirmed and further work will be necessary to provide support for this hypothesis.

The expression of HT7 and EF2, may be explained by the particular characteristics of lesion chondrocytes. However, the remaining two genes examined in this study, Ex-FABP and B-cadherin are, surprisingly, upregulated in TD. This contradicts the hypothesis that the lesion consists of mainly prehypertrophic chondrocytes [6,14], that do not express the genes associated with hypertrophy [18]. Many of the alterations in gene expression associated with the TD lesion that have been reported concern proteins associated with the extracellular matrix of growth plate chondrocytes. There are numerous examples of matrix molecules and growth factors associated with hypertrophy that show reduced levels of expression in the lesion. These include: proteoglycans [8,50], osteonectin [9], type X collagen [7,8,51], osteopontin and bone sialoprotein [18], basic fibroblast growth factor [12] and transforming growth factor β-3 [10,11,50]. However, it is evident from the results presented here, particularly for Ex-FABP and B-cadherin, that the phenotype of TD lesion chondrocytes does not merely reflect the absence of fully hypertrophic cells. Thus, although many of the changes in gene expression in dyschondroplasia documented to date are linked to the process of hypertrophy, there may be other developmental defects that influence the aetiology of TD. One possibility is that impaired vascularisation of the TD growth plate may be involved in lesion formation. It has been demonstrated that the lack of calcification in the TD lesion is due to impaired formation of functional matrix

vesicles [36], and it was suggested that this is due to reduced availability of mineral ions caused by an inadequate blood supply. This failure of vascularisation may be due to a defect in the metaphyseal vessels [52,53], or due to an absence of the angiogenic signals necessary for capillary invasion to occur [54,55]. In this context, it is interesting to note that the production of angiogenic factors in the growth plate is localised predominantly to the hypertrophic zone [56–59], which contains precisely those cells that appear to be absent from the lesion. Whatever the cause, the absence of systemic factors, minerals and nutrients from the TD growth plate is likely to perturb the normal expression pattern of a number of genes, including B-cadherin and Ex-FABP.

4. Conclusions

The results presented here suggest that the formation of the dyschondroplastic lesion is not merely due to the impaired terminal differentiation of lesion chondrocytes and that other developmental defects occur that result in the TD phenotype. Further studies will be necessary to provide insight into the precise nature of the condition.

Acknowledgements

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