The helix-loop-helix transcription factors Id1 and Id3 have a functional role in control of cell division in human normal and neoplastic chondrocytes

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Abstract The expression and localization of the helix-loop-helix transcription factors Id1 and Id3, as well as localization of the E12-protein, were studied in cells isolated from human articular cartilage and chondrosarcoma. Serum withdrawal down-regulated Id1 and Id3 expression in chondrocytes but not the Id1 expression in chondrosarcoma cells. Antisense oligonucleotides directed against Id1 and Id3 decreased BrdU labeling in both cell types. E12 was localized to the nucleus in chondrocytes and non-confluent tumor cells and in confluent tumor cells, E12 had a cytoplasmic localization. This study suggests a functional role for Id1 and Id3 in the control of proliferation and differentiation of cartilage.

Key words: Id; E12; Articular chondrocyte; Chondrosarcoma; Differentiation; Proliferation

1. Introduction

Cell differentiation is related to a decrease in cell proliferation in normal tissues but in tumor cells, loss of differentiated features is combined with uncontrolled proliferation. However, the links between these two events in tumors are unknown, as are most of the events leading to differentiation in normal tissues. The transcription factors family of helix-loop-helix (HLH) proteins has been found to be important for cellular differentiation and thus involved in the developmental control of gene expression. The proteins are divided into different classes; class A consists of the ubiquitously expressed E-proteins, E12 and E47, which are alternately spliced products from the E2A gene [1]. These proteins form heterodimers with the tissue restricted class B proteins, e.g. MyoD in muscle [2]. The heterodimer is formed through the HLH domain of the proteins and the complex is then capable of binding to a specific enhancer element in the DNA, known as the E-box consensus sequence (CANNTG). The basic amino acid region next to the HLH motif mediates the DNA binding.

Another member of the HLH family is a group of proteins called Id (inhibitor of differentiation/DNA binding). These proteins lack the basic amino acid domain. Thus, heterodimers formed by Id and basic HLH proteins will be inactive and no transcriptional activation will appear [3]. Today, there are four known human Id proteins: Id1 [4], Id2 [5], Id3 [6,7] and Id4 [8]. These proteins have highly homologous HLH domains, but homology is low outside this region. For the Id1 gene, an alternative splicing form has been demonstrated in both mouse and human [9–11]. However, the functional role of this non-spliced gene product is still unknown. The wide expression of especially Id1 and Id3 in different cell types suggests an important role and it has been proposed that Id proteins function as general inhibitors of terminal differentiation and thus in control of cell growth. These ideas are supported by the finding that the Id coding genes are expressed in different kinds of tumor cells [6,11,12].

One tissue where differentiation control of HLH proteins has been extensively studied is in muscle development where the proteins have been held responsible for the muscle-specific differentiation from mesenchymal stem cells [2]. The differentiating pathway in cartilage, another tissue of mesenchymal origin, is still unclear. In addition, little is known about the factors and events leading to the formation of chondrosarcoma, a malignant chondrogenic tumor. In an attempt to better understand the molecular interactions between differentiation, dedifferentiation and proliferation in cartilaginous tissue, we have focused on Id1 and Id3 and investigated their expression, at both genetic and protein level, and their protein localization. The functional role of Id1 and Id3 in cultured human articular chondrocytes and in chondrosarcoma cells has been studied with antisense experiments. In addition, one E-protein was also included in this study in order to give a somewhat extended picture of the function of the Ids. Therefore, the cellular localization of E12 was investigated in cultured cells.

2. Materials and methods

2.1. Cell culture

Tumor cells were obtained from 9 cases of chondrosarcoma of different histological grades. There were one grade I–II (K12), four grade II (K5, K6, K8, K9), two grade II–III (K1, K2) and two grade III (K7, K11). K5 and K6 correspond to the cell lines FS090 and 105KC, respectively, both kindly donated by Dr. J. Block. The remaining cell cultures were generated from tumors surgically excised at the Department of Orthopedics, Sahlgrenska University Hospital, Gothenburg, Sweden. In addition, articular chondrocytes (TC) from one of the tumor patients as well as articular chondrocytes (C1–C13) obtained during cartilage transplantation from a total of 13 other patients free from tumors were cultured and analyzed in the same

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Abbreviations: HLH, helix-loop-helix; Id, inhibitor of differentiation/DNA binding; FCS, fetal calf serum; RPA, RNase protection assay; RT-PCR, reverse transcriptase polymerase chain reaction; PBS, phosphate-buffered saline; DTT, dithiothreitol; TBS, Tris-buffered saline; BrdU, 5-bromo-2-deoxyuridine

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way. Monolayer cultured cells were grown in Dulbecco's MEM Nut Mix F-12 (Life Technologies, Paysley, Scotland, UK) supplemented with amphotericin B (2 μg/ml), gentamycin sulfate (50 μg/ml), L-ascorbic acid (50 μg/ml) and L-glutamine (2 mM). To this medium 10% fetal calf serum (FCS, Harlan Sera-Lab, Crawley Down, UK) and 1% Ultroser (Life Technologies) was added. This medium is referred to in the text as serum containing medium. Prior to RNA or protein extraction, cells were trypsinized and rinsed in phosphate-buffered saline (PBS, 0.01 M, pH 7.5). In a first series of experiments, cells were cultured to confluence at serum levels as described. In a second round of experiments, cells from a smaller group of the patients in the study were grown to confluence and then starved by complete serum withdrawal for 24 h before harvesting.

2.2. RNase protection assay

Total cellular RNA was prepared using the RNeasy total RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Using RT-PCR and subsequent cloning into PCRII vectors (Invitrogen, Leek, the Netherlands), riboprobes of 298 bp, 245 bp and 354 bp for human Id1, Id1.25 and Id3, respectively, were generated. The probes were used in the ribonuclease protection assay (RPA) with the RPAII-kit (Ambion, Austin, TX, USA). Briefly, total RNA (7 μg) from cultured articular chondrocytes and chondrosarcoma cells was hybridized overnight at 45°C with [γ-^{33}P]dUTP (Dupont NEN, Bad Homburg, Germany) labeled Id1 and Id3 probes or the Id1.25 probe. RNase digestion was carried out at 37°C for 30 min. As a negative control, the probe was hybridized with 7 μg yeast tRNA. ^{33}P-labeled *Hae*III DNA (Promega, Madison, WI, USA) was used as a molecular size marker.

The RNase protected fragments were separated by electrophoresis through a 6% polyacrylamide gel (Novex, San Diego, CA, USA). The gels were dried and exposed to Phosphor Imager screens. The screens were developed on a Phosphor Imager (Molecular Dynamics, USA).

2.3. Western blot

Total protein extracts were prepared by sonicating cell pellets in 1 mM dithiothreitol (DTT). The protein concentration was determined using the Protein assay kit (Bio-Rad, Hercules, CA, USA). An equal volume protein/DTT and 2× Novex sample buffer were mixed and 20 μg of proteins were loaded in each lane of a 14% Tris-glycine gel. The proteins were size-fractionated on the gel and then electrophoretically transferred onto a nitrocellulose membrane (Novex). The membrane was blocked overnight in 10% dry milk and 0.1% Tween 20 in Tris-buffered saline (TBS) and probed with one of the following polyclonal rabbit antibodies: anti-Id1, anti-Id3 or anti-E12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), all diluted 1:250. After washing in 0.5% Tween 20 in TBS (TBS-T) the membrane was incubated with anti-rabbit Ig, horseradish peroxidase linked F(ab′)$_2$ fragment (Amersham, Buckinghamshire, UK), diluted 1:10 000. The membrane was washed in TBS-T and TBS, and finally the immunoreactive proteins were visualized with ECL Western blotting detection system (Amersham).

2.4. Immunohistochemistry

Chondrosarcoma cells (K2, K5–K9 and K12) and articular chondrocytes (TC, C1, C3, C8–C10) were cultured on Lab-Tek 8 chamber slides (Nunc, Naperville, IL, USA) at approximately 1×10^4 cells/chamber. The cells were grown to confluence under the same conditions as above. The slides were washed in PBS, fixed in cold acetone for 10 min and stored at −70°C. Before use the slides were blocked with 3% FCS (Harlan Sera-Lab) and 3% goat serum (Dako, Glostrup, Denmark) in PBS. The same primary antibodies as above were used, diluted 1:50, and incubated overnight at +4°C. Normal rabbit immunoglobulin (Dako) was used as a negative control. The ABC kit (Vektor Laboratories, Burlingame, CA, USA) were then used according to the manufacturer's instructions, with a biotinylated goat-anti-rabbit secondary antibody diluted 1:200. The slides were dehydrated and mounted with Pertex (Histolab Products, Göteborg, Sweden) and then examined with a light microscope (Nikon, Japan).

Immunohistochemistry was also performed on frozen sections of one of the tumor tissues (K8). This was done in order to control that all three kinds of proteins were expressed in vivo as well as in cultured cells. Basically the same protocol as above was used, with a microwave treatment of the section included before the blocking procedure.

2.5. BrdU-labeling

Articular chondrocytes (C11–C13) and chondrosarcoma cells (K1, K2, K5 and K12) were cultured on 4-well Nunc Lab-Tek chamberslides (Nunc) at approximately 20 000 cells/chamber. The cells were grown under the same conditions as above. After 24 h, the non-confluent cells were subjected to complete serum withdrawal. Twenty-three hours later, antisense phosphorothioate oligonucleotides covering the translation starts of Id1 (5′ GCG ACT TTC ATG ATT CTT GG-3′) or Id3 (5′-CAG CGC CTT CAT GCT GGG GAG-3′) or a random oligonucleotide were added to the medium at a final concentration of 100 μg/ml. After one hour, FCS and Ultroser were added to the media to the final concentrations 10% and 1%, respectively. The cells were then allowed to grow for an additional 24 h before labeling with 5-bromo-2-deoxy-uridine (BrdU), using the BrdU Labeling and Detection kit 1 (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. A minimum of about 150 cells were counted in each experiment (cells in 10 visual areas of a chamber) and the values are given as means ± S.E.M. The statistical significances of differences between means were calculated according to Student's *t*-test for dependent samples (Statistica for Windows 5.1, Stat Soft, Tulsa, OK, USA).

3. Results

3.1. Id1 and Id3 mRNA and protein expression in articular chondrocytes and chondrosarcoma cells

A total of 9 chondrosarcoma cell lines, representing different histological grades, and 11 cultures of normal chondrocytes, obtained from patients during cartilage transplantation, were studied in separate experiments. The cells were either cultured in serum containing media until confluence or starved with complete serum withdrawal 24 h before harvesting. Expression of the Id1 and Id3 genes and proteins were studied by RPA and Western blot, respectively (Table 1).

In RPA, Id1 and Id1.25 mRNA was protected as a 298- and a 245-bp band, respectively, and Id3 mRNA as a 354-bp band. In cultures with serum containing medium, Id1 was expressed in all cells studied (Fig. 1A). The level of Id1 mRNA expression was substantially higher in chondrosarcoma cells compared to normal chondrocytes. However, after serum withdrawal, Id1 expression was abolished in normal chondrocytes but was still present in the chondrosarcoma cells (Fig. 2A). The expression of Id1.25 was only studied in cells cultured at normal serum level. A band representing Id1.25 was detected in cells from three of the high malignancy grade chondrosarcomas (K1, K2 and K7) but not in articular chondrocytes. Id3 showed a similar mRNA expression in tumor cells and articular chondrocytes, and the expression was not dependent on the serum level in the culture media (Figs. 1A and 2A).

Table 1
Expression of Id mRNA and protein in cultured cells

	RPA			Western blot	
	Id1	Id1.25	Id3	Id1	Id3
Experiment 1					
Cells cultured under serum conditions					
Tumor	9/9	3/8	9/9	9/9	5/9
Normal	3/4	0/4	4/4	8/8	2/8
Experiment 2					
Cells subjected to serum withdrawal					
Tumor	5/5		5/5	5/5	0/5
Normal	0/3		3/3	0/5	0/5

Ratios are the number of cell lines expressing mRNA or protein over the number of cell lines screened.

Western blot was used to examine the protein expression of Id1 and Id3. The 16-kDa Id1 protein was generally more strongly expressed in chondrosarcoma cells than in normal chondrocytes, when the cells had been cultured in serum containing media until harvesting (Fig. 1B). In most cells, the expression of mRNA corresponded to the protein level. The tumor cells K6 and K7, derived from a grade II and a grade III chondrosarcoma, respectively, both showed a strong expression of Id1 mRNA, but demonstrated a weak, if any, Id1 protein expression. In addition, an extra band was also visible slightly above 16 kDa in most cells. This band may represent the non-spliced, Id1.25 mRNA protein product.

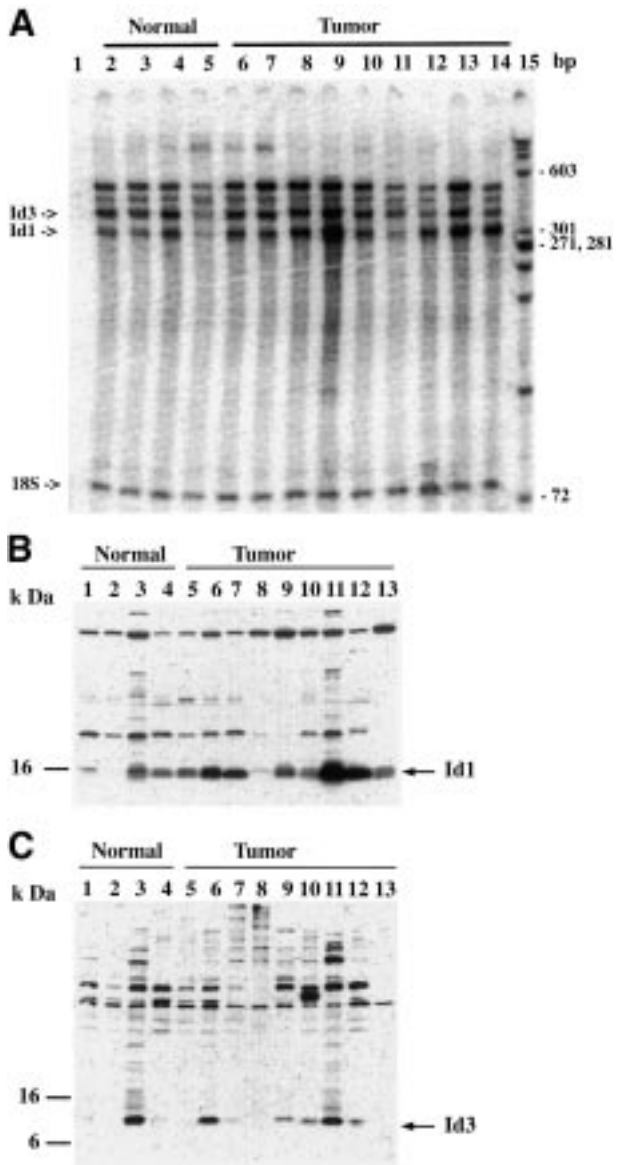

Fig. 1. Expression of Id1 and Id3 in cells cultured in normal serum level. A: RNA expressions shown by RNase protection assay. The bands corresponding to Id1 and Id3 are shown by arrows. A probe hybridizing to 18S was used as an internal control for equal loading onto the gel. Lane 1: tRNA serving as a negative control; lanes 2–5, articular chondrocyte mRNAs from patients C1, C2, C3 and TC, respectively; lanes 6–14: tumor cell mRNAs from patients K9, K8, K5, K6, K11, K12, K1, K2 and K7, respectively; lane 15: *Hae*III DNA molecular size marker. Western blot was used to study the protein expression of Id1 (B) and Id3 (C). Lanes 1–4: Articular chondrocytes from patients C1, C2, C3 and TC, respectively; lanes 5–13: tumor cell proteins from patients K9, K8, K5, K6, K11, K12, K1, K2 and K7. The expected bands are indicated by arrows.

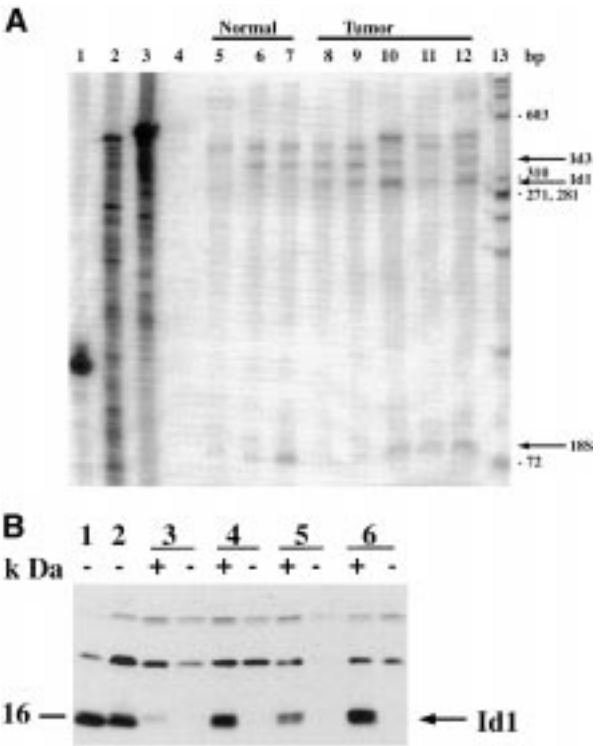

Fig. 2. Expression of Id1 and Id3 in cells cultured with complete serum withdrawal 24 h before harvesting. A: RNA expression of Id1 and Id3 shown by RNase protection assay. Lane 1: 18S probe; lane 2: Id1 probe; lane 3: Id3 probe; lane 4: tRNA used as a negative control; lanes 5–7: articular chondrocyte mRNA from patients TC, C8 and C9, respectively; lanes 8–12: chondrosarcoma cell mRNA from patients K2, K5, K8, K12 and K9, respectively; lane 13: *Hae*III DNA size marker. B: Protein expression of Id1, shown by Western blot. Comparison between articular chondrocytes cultured with and without serum withdrawal. Lanes with protein from cells cultured with full serum level are indicated with + and lanes with proteins from cells that were subjected to 24-h serum withdrawal before harvesting are indicated by −. Lanes 1, 2: Chondrosarcoma cell proteins from patients K7 and K1, respectively; lanes 3–6: articular chondrocyte proteins from patients C4, C5, C7 and C6, respectively.

After 24 h serum withdrawal, the protein expression of Id1 was only present in the tumor cell lines (Fig. 2B).

A variation of Id3 protein expression was detected by Western blot in the non-starved cells (Fig. 1C), but after serum withdrawal, the protein was absent in both normal cells and tumor cells.

3.2. *Cellular localization of Id1, Id3 and E12 proteins in articular chondrocytes and chondrosarcoma cells*

Cells were cultured on chamberslides with serum containing media until 24 h post confluence. Immunohistochemistry was used to study the cellular localization of the Id1, Id3 and E12 proteins. Id1 staining was predominantly cytoplasmic. Id3 staining was both cytoplasmic and nuclear. The E12 protein showed nuclear staining both in preconfluent and confluent cells. In chondrosarcoma cells, on the other hand, the nuclear staining seen in preconfluent areas was not demonstrated in confluent cells (Fig. 3).

Immunohistochemistry on frozen sections of tumor tissue

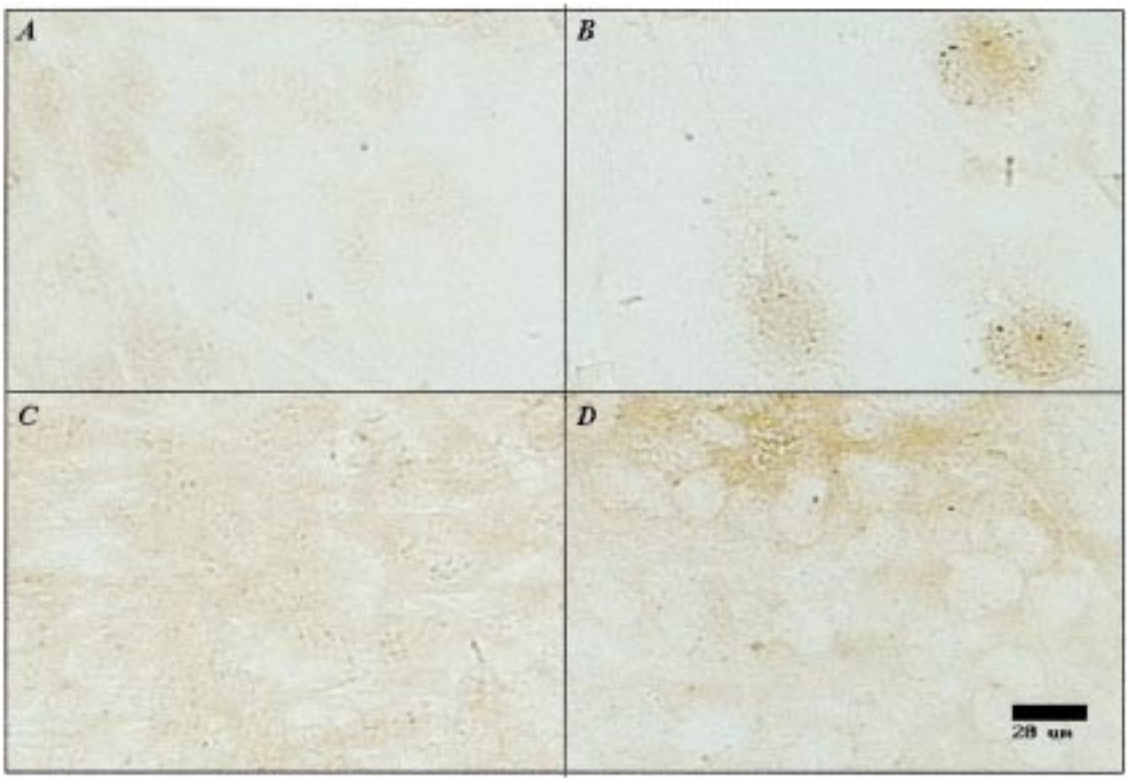

Fig. 3. Cellular localization of E12 detected by immunohistochemistry. Normal articular chondrocytes from patient C8 are seen in the left panels (A and C) and chondrosarcoma cells from patient K9 are shown to the right (B and D). Sparse tumor cells (B) show nuclear staining as well as both sparse and confluent articular chondrocytes (A and C), while cytoplasmic staining and almost no nuclear staining are detected in confluent chondrosarcoma cells (D).

showed a weak staining for all three proteins, indicating that expression is not restricted to cultured cells (data not shown).

3.3. *Effect of antisense stimulation of Id1 and Id3 in articular chondrocytes and chondrosarcoma cells*

To study the function of Id1 and Id3, a series of antisense experiments was carried out. To non-confluent cells cultured in chamberslides, different antisense oligonucleotides were added and the DNA synthesis was determined by BrdU labeling. Besides the antisense oligonucleotides of Id1 and Id3, a random oligonucleotide was also used in order to see that the addition of oligonucleotides was non-toxic to the cells. Similar values of BrdU incorporation were obtained with random oligonucleotides added and without any oligonucleotide at all. The BrdU labeling was found to be significantly lower when Id1 or Id3 antisense oligonucleotides had been added to the culture medium than when the random oligonucleotide was added (Fig. 4). In chondrosarcoma cells, the BrdU incorporation was reduced from 43% to 39% with Id1 antisense oligonucleotide and to 26% with Id3 antisense oligonucleotide. In chondrocytes, the reduction was from 39% to 33% for Id1. After addition of Id3 antisense oligonucleotide to the articular chondrocytes, as well as to the low-graded chondrosarcoma cells (K12), most cell boundaries seemed to be disrupted and cell counting was impossible.

An additional experiment was also performed on starved cells. No BrdU incorporation was noted after serum withdrawal of chondrocytes, while a mean value of 9% labeled cells was seen among the chondrosarcoma cells.

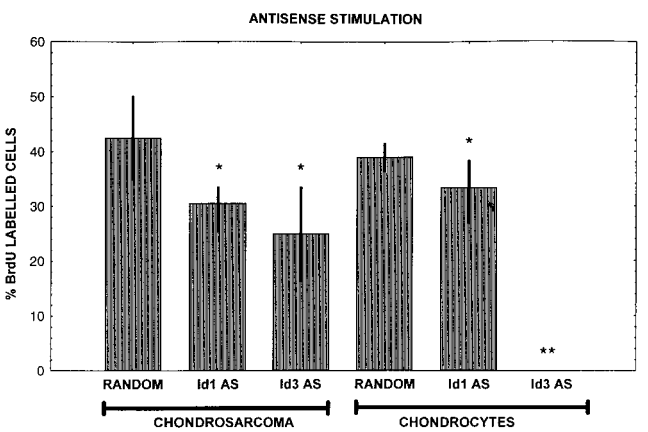

Fig. 4. Effect of Id1 and Id3 antisense oligonucleotides on cultured cells. The mean values of % BrdU-labeled cells and the standard error of the mean (S.E.M.) are shown.

4. Discussion

This study has been focused on the two HLH transcription factors Id1 and Id3 since these have a general expression in different tissues and have been found in other tissues of mesenchymal origin. We wanted to investigate the functional role for Id1 and Id3 in cartilage and how expression and localization of these transcription factors were affected by different grades of differentiation in cartilage. Since it is difficult to obtain chondrocytes with different stages of differentiation,

the chondrosarcoma cells were chosen to represent the less differentiated stage.

The expression of the Id proteins was examined at the genetic and protein level under different culture conditions. After a first series of experiments with cells cultured under serum condition, a smaller series of experiments was performed with cells subjected to complete serum withdrawal for 24 h before harvesting, to see whether the Id pathway was activated regardless of serum supplementation of the culture media. In the case of Id1, normal chondrocytes expressed both mRNA and protein to a varying degree when cultured in serum containing medium. However, when the cells were subjected to serum withdrawal, mRNA as well as protein were absent. This finding is consistent with the function of Id as an inhibitor of differentiation. A lack of differentiation promotes further dividing, i.e. Id1 directly or indirectly stimulates cell division. Antisense experiments showed a significantly lower cell proliferation when antisense for Id1 was added to either chondrocytes or chondrosarcoma cells. This shows that Id1 supports a proliferative function also in cells of cartilaginous origin. When the chondrocytes are starved, components in the culture media which are a prerequisite for cell division disappear and the cells stop dividing. Thus, Id1 is no longer needed to stimulate cell division since the basic needs for proliferating cells are absent. In this manner, serum supplementation stimulates the expression of Id1, either directly through proliferation or through another mechanism which in turn will lead to cell division. The tumor cells, on the other hand, expressed both Id1 mRNA and protein irrespective of serum conditions. Even if chondrosarcoma generally is a very slowly growing tumor and often requires high serum levels in the media in order to speed up growth rate, this tumor has an ability to express a proliferating factor, which inhibits differentiation and stimulates cell division, without any serum stimulation at all. The strong Id1 protein expression even after serum withdrawal indicates an autocrine or paracrine stimulation which induces a less differentiated state of the tumor and indirectly stimulates proliferation. The BrdU-labeling experiments showed that chondrosarcoma cells were able to undergo cell division even without serum.

Concerning Id3, the protein expression was dependent on serum in both chondrocytes and chondrosarcoma cells with no detectable expression after serum withdrawal. Since chondrosarcoma cells were shown to divide even without serum, the Id3 protein expression seem to be influenced by factors other than those required for cell division. However, both kinds of cells had a weak to moderate expression of Id3 mRNA even after starvation. One interesting observation was that the Id3 antisense oligonucleotide added to articular chondrocytes or to low graded chondrosarcoma cells disrupted the cell boundaries due to a general toxic effect or through induction of apoptosis. Enforced expression of Id3 has been found to induce apoptosis in serum-deprived rat embryo fibroblasts [13]. This data together with ours suggests that the cell needs a balanced expression of Id3 to function properly. Id3 seems to have a basic function in normal cartilage. In highly malignant tumor cells a significant reduction was seen in cell proliferation when Id3 antisense was added, instead of cell destruction. This may indicate that alteration of a regulatory pathway including Id3 is a late event in cartilage tumor formation. Studies on NIH 3T3 fibroblasts have shown similar results as ours. When the cells were cultured in low serum levels, mRNA expression of both Id1 and Id3 were reduced and a similar antisense experiment also showed reduced proliferation [14].

Id1.25 was only expressed in a third of the tumor cells at the mRNA level. At the protein level, however, a double band around 16 kDa was seen in most cells, both from neoplastic and normal origin. There is no evidence that the band of slightly higher molecular weight really represents Id1.25, and any conclusion regarding the protein expression of this altered splice form should be drawn with caution. In this study, we have chosen to simply regard both bands as unspecified forms of Id1.

The cellular localization of Id1, Id3 and E12 was evaluated with immunohistochemistry. Mainly, cytoplasmic staining of Id1 was seen, while Id3 generally had both cytoplasmic and nuclear staining.

In the case of E12, nuclear staining was detected in all normal cells, with the exception of the articular chondrocytes derived from one of the tumor patients. In these cells, cytoplasmic staining was more frequent. One factor worth noticing, however, is the higher age of this patient (60 years) as compared to the patients C1–C10 (range from 23 to 43 years). The above results correlate well with previous findings [15]. In the chondrosarcoma cells, however, nuclear staining occurred in pre-confluent areas, while in confluent areas cytoplasmic staining was seen. This observation suggests an explanation for the continuous proliferation seen in tumor cells. When the cells are confluent, their proliferation normally should be inhibited. Differentiation, on the other hand, is able to start when the nuclear E12 binds to DNA. In tumor cells, E12 was absent in the confluent cell nuclei. This probably will affect the E12-dependent differentiating pathway which cannot start. If differentiation signals lead to decreased proliferation, rather than the opposite, it seems likely that proliferation will continue as long as E12 is missing in the nucleus.

In conclusion, members of the helix-loop-helix transcription factor family seem to be important elements in the delicate interactions between differentiation and dedifferentiation in cartilaginous tissue. Still, our knowledge is fragmentary of the regulatory pathway involving the HLH proteins. Our findings support the theory of an unknown HLH transcription factor active in cartilage.

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