Abstract. Background: By mediating local cell-cell interactions, the Notch signaling pathway seems to control a variety of processes from cell fate decisions during development, to stem cell renewal and to differentiation in many adult tissues. Hence, perturbed Notch signaling may be involved both in the development and the spread of cancer. The expression and the functional role of some major components of the Notch signaling pathway in human hepatocellular carcinoma (HCC) are poorly characterized. Materials and Methods: Notch3, HES1, Jagged1 and Delta1 were analyzed both at the RNA and protein levels in the HepG2 liver cell line derived from human HCC. Results: The results of this study demonstrated, for the first time, that both Jagged1 and Delta1 ligands and the downstream effector gene HES1 are expressed in the HepG2 actively proliferating cell line. Moreover, a high expression of Notch3 intracellular domain, indicative of constitutively activated Notch signaling, was the only detectable Notch3 subunit in HepG2. Conclusion: These findings suggest that Notch3 may be involved in mechanisms controlling the differentiation and the spread of HCC and that Notch3 activation may be dependent on both Jagged1 and Delta1 ligands.

Notch genes encode for a family of highly conserved single-pass transmembrane receptors known as Notch 1, 2, 3 and 4. Notch receptors are synthesized as ~300 kDa proteins (pro-Notch) which are then cleaved by a furin-like convertase in the Golgi. This proteolytic event releases an extracellular subunit (NEC~200 kDa) containing many epithelial growth factor repeats and a lin-12 Notch repeat as well as a transmembrane subunit (N\textsuperscript{TM} 97-120 kDa) containing a short extracellular fragment and an intracellular domain (N\textsuperscript{ICD} 65-110 kDa) (1). These non-covalently associated subunits are presented as a heterodimeric functional receptor at the cell surface. Even though ligand-independent activation of the Notch signaling pathway has been described (2), Notch receptors are mainly activated by transmembrane ligands expressed on the surface of neighboring cells. Five ligands of Notch receptors have been described in vertebrates: Delta-like 1, 3 and 4 and Jagged 1 and 2 (3, 4). Upon ligand binding to the extracellular domain of Notch receptors, two sequential proteolytic events occur. First, cleavage takes place twelve amino acids outside the transmembrane domain by metalloproteinase TACE/ADAM17 (5). The resulting Notch COOH-terminal fragment, called NEXT (Notch extracellular truncation), is required for the second cleavage performed by \(\gamma\)-secretase within the transmembrane region. This last proteolytic event releases the Notch intracellular domain (NICD) into the cytoplasm (6). The NICD translocates into the nucleus, binding to the transcription factor CBF1/RBP-Jk transactivating target genes. To date, the HES (hairy/enhancer of split) and HERP (HES-related repressor protein) families of transcriptional repressors are the only known major targets of Notch signaling (7, 8).

By mediating local cell-to-cell communication, the Notch family of receptors controls either cell fate and spread during development or stem cell renewal and differentiation in many adult tissues (9, 10). A perturbed Notch signaling pathway has been described in different human diseases, including cancer. Constitutively active Notch1 and Notch4 polypeptides were found to be involved in human acute T-lymphoblastic leukemia (T-ALL) and mouse mammary tumor development, respectively (11, 12). The induced expression of constitutively active Notch4 has been described to promote the expansion of hematopoietic stem...
cells in vitro (13). These observations suggest that unregulated NICD expression prevents differentiation, favoring malignant transformation. On the other hand, constitutive activation of Notch1 may function as a tumor suppressor in small cell lung cancer cells, in prostate cancer cells and in mouse skin (14-16) by inducing cell growth arrest.

Epidemiological evidence indicates that human hepatocellular carcinoma (HCC) is one of the most prevalent neoplasms worldwide. The molecular mechanisms controlling the differentiation and the spread of HCC are still largely unknown. The Notch1 signaling pathway is activated during rat liver regeneration and overexpression of Notch1 has been found to inhibit the growth of HCC cells in vitro and in vivo (17). Notch3 has recently been described to be involved in bile duct development (18) but, to date, there are no studies concerning Notch3 expression and function in HCC.

The aim of the present study was to investigate the expression of some major components of the Notch signaling pathway, including Notch3, Jagged1, Delta1 and the downstream effector gene, HES1, in the HepG2 tumor cell line.

Materials and Methods

Cell lines. The HCC HepG2 cell line was obtained from the American Type Culture Collection (HB-8065, ATCC, Rockville, MD, USA) and was maintained in Eagle's minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all reagents from ATCC) at 37°C in a 5% CO2 incubator.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was isolated from the HepG2 cell line by TRIzol (Invitrogen, Paisley, Scotland), according to the manufacturer's instructions. Four µg of total RNA were treated with DNase I (Invitrogen) to eliminate contaminating genome DNA. RT was performed in 30 µl of reaction mixture including: 1X RT buffer, 0.4 mM dNTPs, 5 mM dithiothreitol (DTT), 0.5 µM oligo dT, 3 µM random primers, 240 U Superscript II (all reagents from Invitrogen). The RT reaction was carried out at 42°C for 1 h, followed by 5 min at 95°C to inactivate the enzyme. Hes1, Jagged1 and Delta1 gene expressions was assessed by real-time PCR using the iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Notch3 gene expression was assessed using both real-time and conventional endpoint PCR amplification.

For real-time PCR, all transcripts were amplified in 25 µl of reaction using 1 µl of cDNA, 300 mM of each primer and the SYBER Green Supermix (Bio-Rad). Standard curves were generated by making appropriate dilutions of samples of cDNA synthesized from human liver tissues. The real-time products were loaded on agarose gel to rule out aspecific amplifications. Conventional PCR was performed with Herculase Taq Polymerase (Stratagene, La Jolla, CA, USA) as described by the company and the PCR product was resolved on 1% agarose gel containing ethidium bromide. The primer sequences and the annealing temperature are listed in Table I.
Protein extraction. The cultured HepG2 cell line was dissolved in lysis buffer containing 10 mM Tris-HCl pH 7.4, 2.5 mM MgCl₂, 1% TritonX 100, 1 mM DTT, 0.1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Sigma Chemical Co., St. Louis, MO, USA). The lysate was then centrifuged at 4°C, 15,000 x g, for 15 min and the supernatant was assayed for the protein concentration using the Bio-Rad protein assay (Bio-Rad).

SDS-PAGE and Western blot analysis. Fifty μg of proteins were boiled at 95°C for 10 min in 1X SDS-loading buffer (65 mM Tris-HCl, pH 7.5, 65 mM 2-mercaptoethanol, 1% SDS, 10% glycerol and 0.003% bromophenol blue), resolved by 5% or 8% polyacrylamide gels and blotted on nitrocellulose membranes (Hybond C Extra, Amersham Pharmacia, Little Chalfont, UK). The membranes were stained with Red Ponceau solution (Sigma), blocked in 5% non-fat dry milk for 50 min in phosphate buffered saline (PBS) and then incubated with the appropriate primary antibody. The primary antibodies and dilutions used are listed as follows: anti-Notch3 polyclonal antibody (sc-5593, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:400, anti-Notch3 polyclonal antibody (BC4), kindly provided by Dr. A. Joutel (Faculty of Medicine, Lariboisiere, Paris) 1:10,000, anti-Delta1 polyclonal antibody (sc-9102, Santa Cruz) 1:200, anti-Jagged1 polyclonal antibody (sc-8303, Santa Cruz) 1:100.

After repeated washing in PBS containing 0.1% Tween 20 (PBST), the membranes were incubated with anti-mouse or anti-rabbit HRP-conjugated secondary antibodies using the EnVision dextran polymer visualization system (DAKO, Denmark). The membranes were washed and autoradiographies were obtained using a chemiluminescence reaction (ECL reagents, Amersham). Digital images of the autoradiographies were acquired with a scanner (Fluor-S MultiImager, Bio-Rad) and signals were quantified using specific densitometric software (Quantity-one, Bio-Rad) in absorbance units after light calibration with a reference autoradiography.

Immunocytochemistry. The HepG2 cells were seeded on sterilized cover-slips and fixed in 4% paraformaldehyde. The cells were then permeabilized in PBS containing 0.1% of saponin and incubated with normal goat serum at room temperature for 30 min. Notch3 protein localization into the cell was assessed with the same antibody used in Western blot (Santa Cruz), followed by an HRP-rabbit EnVision system with Vector Red (Vector Laboratories, Burlingame, CA, USA) as chromogen. The cells were then counterstained with Mayer’s hematoxylin and mounted with DPX (BDH Chemical, Poole, UK). Negative controls were obtained by omitting the primary antibody.

Results

Semi-quantitative real-time PCR. To investigate Notch3, Jagged1, Delta1 and HES1 gene expressions, semi-quantitative real-time PCR was performed on RNA extracted from the HepG2 cell line. The real-time PCR efficiency was almost identical for the genes analyzed. The cycle thresholds (Ct) were as follows: CtNotch3 = 33, CtDelta1 = 34, CtJagged1 = 20, CtHES1 = 26, suggesting that the levels of Notch3, Jagged1, Delta1 and HES1 gene expressions were significantly different. Faint bands were observed for Notch3 and Delta1 real-time PCR products resolved on agarose gel. A high Notch3 expression was observed with conventional PCR where a proper Taq Polymerase (Herculase) efficient with GC-rich templates, such as the Notch genes, was used (Figure 1).

Protein expression. Notch3 protein expression, determined by immunoblotting, was observed as a band corresponding to...
to the molecular weight of 76 kDa, matching the cleaved Notch3 intracellular domain (NICD). Conversely, both the 97-kDa and 300 kDa bands, corresponding to the Notch3 transmembrane subunit and to the Notch3 full-length receptor, respectively, were not detected. The same results were obtained by using two different antibodies, as described above (Figure 2). This evidence was supported by immunocytochemical results which showed a strong staining for Notch3 in the cytoplasm and in a few nuclei (Figure 3). On the other hand, no positive staining was observed at the membrane level of HepG2 cells, suggesting the complete lack of the transmembrane Notch3 subunit.

Both the Jagged1 and Delta1 ligands were detected by immunoblotting and Jagged1 was expressed three times more than Delta1 (Figure 2). The difference in protein levels between Jagged1 and Delta1 paralleled their different gene expressions as evaluated by real-time PCR. Notch3 protein expression correlated with the mRNA level detected by conventional PCR.

Discussion

By mediating a balance between cell proliferation and differentiation, Notch signaling has been suggested to be involved in malignant transformation (19). The role of Notch in tumorigenesis is still largely unknown and it seems to be tumor type-dependent (12, 15, 17).

Notch3 has been described to be involved in different processes, including bile duct development and vascular smooth muscle cell (VSMCs) growth (18, 20). Mutations in the Notch3 receptor are involved in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (21). Altered Notch3 seems to allow the disruption of early thymocyte differentiation, favoring the development of T-cell leukemia in the mouse (22). To date, little is known about Notch3 expression and function in human cancer.

In the present study, the expression of some major components of the Notch signaling pathway, including Notch3, Jagged1, Delta1 and the downstream effector gene, HES1, were analyzed in the HepG2 tumour cell line. At the protein level, the absence of a 300-kDa band, corresponding to the Notch3 full-length receptor, could be explained in Western blot by the non-covalent association between the extracellular subunit and the transmembrane subunit destroyed during protein extraction. On the other hand, both antibodies, specific for epitopes within the carboxy-terminus of Notch3, failed to detect the Notch3 transmembrane subunit, suggesting the complete lack of the receptor on the cellular membrane. This observation was in line with immunocytochemical results which failed to show positive staining at the cellular membrane level. A high expression of endogenous NICD, indicative of active Notch signaling, was the only detectable Notch3 subunit in the HepG2 cell line. At the immunocytochemical level, strong staining for Notch3 was observed in the cytoplasm and in a few nuclei, suggesting that a very low amount of NICD migrates into the nucleus to activate target genes. It has recently been described that different Notch receptors can have opposing effects in a single tumor type (23). This evidence suggests that a cross-talk among different Notch receptors could exist, giving a possible explanation for the large amount of activated Notch3 kept in the cytoplasm, where it is probably poly-ubiquitylated and degraded by proteasome (24). In order to detect Notch signaling activity, HES1 gene expression was investigated by real-time PCR. HES1 was found to be expressed in the HepG2 cell line and its expression could be regulated, at least in part, by NICD3 in the nucleus.

We have no data to demonstrate that Notch3 activation is induced by Delta1 or Jagged1 ligands, both expressed in these cells. Even though ligand–receptor activation by Jagged versus Delta is modulated by the glycosylation of residues within the epithelial growth factor repeats of the Notch extracellular domain (25), the higher protein levels of Jagged1 than Delta1 observed imply that Notch3 persistent activation could be mediated by Jagged1.

This study provides the first evidence that some major components of the Notch signaling pathway, including Notch3, Jagged1, Delta1 and the downstream effector gene HES1, are expressed in the HepG2 tumor cell line. A high expression of endogenous NICD, indicative of persistent Notch signaling, could be necessary for malignant liver cell proliferation; the same result was obtained in the HCC cell line Hep3B (data not shown). Our hypothesis is supported by a previous study showing that the constitutive activation of the Notch3 receptor in VSMCs induces a persistent state of cell cycle progression by mediating suppression of the cell cycle inhibitor p27kip (20). Taking our results into consideration, along with those from previous studies, it can be supposed that deregulation of Notch receptor activity may be involved both in the differentiation and spread of HCC. In order to investigate whether some genes regulated by Notch3 are involved in such processes, other studies including interference RNA against Notch3 will be performed.

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References


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