Neurotensin Receptors in Adeno- and Squamous Cell Carcinoma

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Abstract. *Background: Peptide receptors seem to be good markers for receptor targeting because of their overexpression in human cancer. Understanding the role of receptors and their cognate ligands, they are currently used for both diagnosis and therapy. Candidates playing a key role in tumor biology are the neurotensin receptors (NTR). The expression of NTR in HT-29 cells (human colon adenocarcinoma cell line), FaDu cells (human squamous cell carcinoma cell line) and in corresponding tumor xenografts on nude mice, was investigated. Materials and Methods: Quantitative RT-PCR of the three receptor subtypes was carried out to study mRNA expression. Receptor protein expression was analyzed by immunohistochemistry with specific antibodies for the three known neurotensin receptors NTR1, NTR2 and NTR3. Results: Analysis of receptor mRNA revealed a strong expression of NTR3 and a weak expression of NTR1 and NTR2 in cultured cells and xenografts. Examining the protein levels, a strong signal for NTR1 was detected in tumor cells and xenografts and only a weak signal was detected for NTR3. Conclusion: Since the receptor protein is targeted in vivo, the enhanced protein expression of NTR1 in xenografts could be a useful tool for molecular targeting with radioligands and for further characterization of the carcinogenic process.*

The significance of peptides and peptide receptors in cancer has become of increasing interest during the past twenty years. As already shown, these receptors are often overexpressed in many primary human cancers compared to their expression in normal tissue (1). Due to their large enrichment in certain tumors, peptide receptors have become increasingly important for clinical use: a) for the targeting of peptide receptors with radiolabeled peptides for early diagnostics, and b) as peptide targets for mediating functional responses of receptors for therapeutic purposes (2).

One family of receptors with a great potential in the field of molecular targeting are the neurotensin receptors (NTR), known to be overexpressed in various tumor cell lines, including small cell lung carcinoma, neuroblastoma, pancreatic and colon cancer (1, 3-6). Three NT receptors, NTR1, NTR2 and NTR3, have been identified to date. NTR1 and NTR2 belong to the family of G protein-coupled receptors with seven transmembrane domains, whereas NTR3 is a single transmembrane domain protein that belongs to a recently identified family of sorting receptors. The biochemical, pharmacological and biological properties of these receptors were reviewed recently (7). Neurotensin (NT), an endogenous tridecapeptide with a wide spectrum of biological activities in the central and peripheral nervous system, mediates multiple effects *via* the NTR subtypes (8-12). NTR have been shown to be internalized after interaction with NT. Several lines of evidence suggest that NT plays a role in cancer due to its stimulating effects on the proliferation of tumor cell lines *in vitro*, including those originating from pancreas, prostate, brain and lung cancer (13-17).

In order to verify the molecular characteristics, both receptor mRNA and receptor protein expression were studied to get a better insight into the disease mechanism. In human tumors mRNA and the protein levels for receptors differ and it is thus important to understand their potential functions in cancer cell growth. This differentiation is dependent on both the tumor environment and cell differentiation.

Sodium butyrate, a short-chain fatty acid naturally present in the human colon modulates a variety of fundamental cellular processes, *i.e.,* inducing cell cycle arrest, differentiation and apoptosis. It has also been suggested that the use of differentiation inducers is an alternative therapeutic approach to conventional tumor therapy (27-30).

The purpose of the present study was to characterize differences in mRNA and protein expression of NTR1,

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Table I. *PCR program protocols.*

cDNA	Initial denaturation	Denaturation	Annealing	Extension	Final extension
NTR-1	94˚C 5 min	94˚C 30 s	57˚C 30 s	72˚C 1 min	72˚C 5 min
NTR-2	94˚C 5 min	94˚C 30 s	58˚C 30 s	72˚C 1 min	72˚C 5 min
NTR-3	94˚C 5 min	94˚C 30 s	58˚C 30 s	72˚C 1 min	72˚C 5 min
18S rRNA	94˚C 5 min	94˚C 30 s	65˚C 30 s	72˚C 1 min	72˚C 5 min

NTR2, and NTR3 under *in vitro* conditions and after transplantation of the cells in nude mice. The HT-29 is an accepted model of human colon adenocarcinomas overexpressing NTR. Considering the cellular and tissue heterogeneity of several tumors, the human squamous cell carcinoma model cell line (FaDu) and the corresponding xenografts were also investigated as a second tumor model in the present study. In addition, whether the expression of NTR1 might be a representative marker for tumor targeting *via* radiolabeling was studied in order to further characterize carcinogenic processes.

Table II. *Sequences of primers used in PCR.*

cDNA	Primer	Primer sequence (5' to 3')	Size
NTR-1	5'	GAGCACAGCACATTCAGCAT	211 bp
NTR-1	5'	GTGCGTTGGTCACCATGTAG	
NTR-2	5'	CAGGTGAATGTGCTGGTGTC	240 bp
NTR-2	5'	GCGCACGTCTTTATGTCTCA	
NTR-3	5'	GTTCTGTCTGCCATGGGTTT	424 bp
NTR-3	5'	AGGTCCAAACACAGGAGGTG	
18S rRNA	5'	GCTGGAATTACCGCGGCTGCT	189 bp
18S rRNA	5'	CGGCTACCACATCCAAGGAAGG	

Materials and Methods

NTR expression studies.

a) Cell culture. HT-29 (ATCC Number: HTB-38) and FaDu (ATCC Number: HTB-43) cells were cultured at 37˚C with 5% CO_2 and 95% air in McCoy medium (HT29) and RPMI medium (FaDu) supplemented with 200 μg/ml penicillin, 200 μg/ml streptomycin and 10% fetal calf serum (Biochrom). For cell differentiation, the HT-29/FaDu cells were incubated in the absence or presence with different concentrations of sodium butyrate (0.1, 0.5, 1, 2 and 4 mM) (Sigma, Taufkirchen, Germany) for four days.

b) Animals and tumor model. The experiments were performed using 7-14-week-old male and female NMRI (nu/nu) mice (Experimental Centre of the Medical Faculty of the University of Technology Dresden). The animal facilities and the experiments were approved according to the institutional guidelines and the German Regulations for Animal Welfare. To immunosuppress the nude mice further, they were whole-body irradiated 2 days before tumor transplantation with 4 Gy (200 kV X-rays, 0.5 mm Cu filter, 1 Gy/min).

FaDu is an established human hypopharyngeal SCC line, kept in high passage by the American Type Culture Collection (Rockville, MD, USA) (18-20). HT-29 is a human colon adenocarcinoma obtained from the German Collection of Microorganisms and Animal Cell Cultures (Braunschweig, Germany). According to a standardized protocol, a stock of cryoconserved tumor pieces was established for both tumor cell lines. For the experiments, source tumors were generated from this stock, cut into small pieces and transplanted subcutaneously into the right hind-leg of anaesthetized mice (120 mg/kg body weight ketamine [intraperitoneal, *i.p.*] and 16 mg/kg xylazine *i.p.*) (20-21).

FACS-analysis. For determination of the degree of differentiation, the cells were harvested in the appropriate manner, washed with PBS and fixed in cold 70% ethanol for at least 30 min. Cells can be left at this stage for several weeks. After washing cells with PBS twice, the cells were stained with 200 μl propidium iodide (50 μg/ml) (Sigma) for 30 min in the dark and were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany). The cell cycle analysis was done with CellQuestPro software (Becton Dickinson).

RNA isolation. For preparation of total RNA from HT-29/FaDu cells and the corresponding xenografts, a "Micro-to-Midi Total RNA Purification System" (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's recommendations.

RT-PCR. RT-PCR was performed using SuperScript One-Step RT-PCR kit from Invitrogen according to the manufacturer's recommendations. For each gene (cDNA) investigated, 35 PCR cycles were performed. The protocols are listed in Table I. All the primers (Table II) were designed from their cDNA (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Metabion, Germany. Agarose gels were monitored by GENE Flash and band densitometry was analyzed with GeneTools software (SynGene Bio Imaging, Cambridge, UK).

Membrane protein purification. For the extraction of membrane proteins, a detergent-based protein purification method was carried out as described previously (21).

Western Blot. Membrane proteins were denatured by boiling at 95˚C for 5 min using 6 x Laemmli sample buffer. Protein samples were electrophoresed on 5% or 10% SDS polyacrylamid gel and subsequently electroblotted onto PVDF membranes for 2 h at constant 10 V. The membranes were blocked in PBS containing 2% (w/v) BSA for 1 h at room temperature and were incubated with primary antibody directed against neurotensin receptor 1, 2

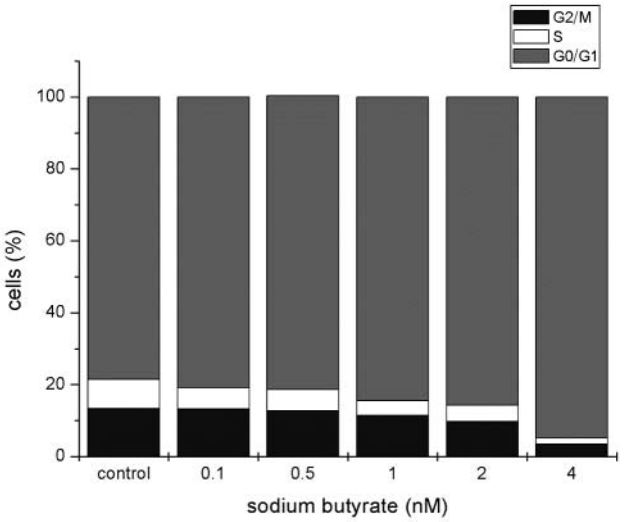

Figure 1. *Flow cytometry showing cell cycle analysis of differentiated HT-29 cells. Differentiation was carried out at different concentrations of sodium butyrate over four days.*

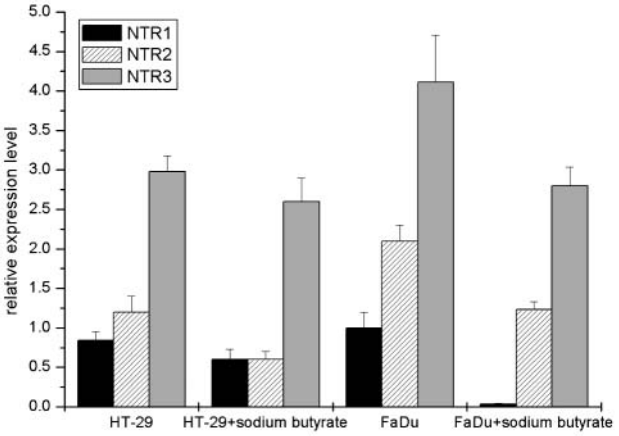

Figure 2. *Quantitative RT-PCR analysis of NTR expression in HT-29 and FaDu cells. NTR expression was normalized to the house-keeping gene 18S rRNA expression. For cell differentiation, HT-29 and FaDu cells were incubated in the presence of 4 mM sodium butyrate for four days. Data are mean±SD (n=5).*

and 3 purchased from Alpha Diagnostic Int. (San Antonio, Texas, USA) overnight at 4˚C. The bound antibody was visualized using a peroxidase conjugated anti-rabbit antibody (Sigma) and by chemiluminescence assay (Pierce, Rockford, IL, USA) and was recorded with Hyperfilm ECL (Amersham Bioscience, Uppsala, Sweden).

Immunohistochemistry. Immunohistochemistry for the NTR receptor was performed on frozen HT-29 and FaDu tumors. Crystat sections (20 μm thick) were fixed with 4% paraformaldehyde and 2% sucrose in PBS for 20 min at 4˚C. They were then incubated with methanol and 2% H_2O_2 (v/v) for 20 min to quench endogenous peroxidase activity. After immobilization with 1% Triton X-100 for 10 min and blocking with 2% BSA in PBS for 1 h, the sections were incubated with primary antibody directed against neurotensin receptor 1, 2 and 3, purchased from Alpha Diagnostic Int., overnight at 4˚C. Section-bound antibodies were detected by incubation with a peroxidase-conjugated anti-rabbit antibody (1:200) (Sigma) for 2 h at room temperature. FAST-3, 3'diaminobenzidine tablets (Sigma) were used as a precipitating substrate for the detection of peroxidase activity. The specificity of labeling was tested by omitting the primary antibody.

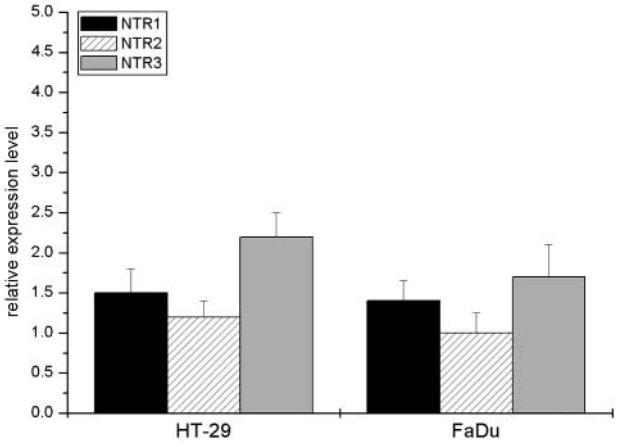

Figure 3. *Quantitative RT-PCR analysis of NTR expression in HT-29 and FaDu xenografts. NTR expression was normalized to the house-keeping gene 18S rRNA expression. Data are mean±SD (n=5).*

Results

Degree of HT-29 and FaDu cell differentiation effected by sodium butyrate. To evaluate the effect of sodium butyrate on the cell cycle, the DNA content was measured by FACS analysis and the relative percentage of the proportions of cells in the G0/G1 (2C DNA content), S (2C-4C) and G2/M (4C DNA content) stages was calculated. The vast majority of the cells with increasing sodium butyrate concentration were in G0/G1 (Figure 1). Surprisingly, the magnitude of butyrate effect on cell differentiation was seen with high sodium butyrate concentrations (4 mM) after four days of incubation. As shown in Figure 1, increasing the sodium butyrate concentration caused an increase in G0/G1 and a decrease in the S and G2/M stages. All data presented here refer to HT-29 cells. The same effects with respect to differentiation magnitude were also observed in the FaDu cells (data not shown in detail).

NTR mRNA expression in HT-29 and FaDu cell lines and corresponding xenografts. The distribution of NTR mRNA was determined by RT-PCR from HT-29 and FaDu cells

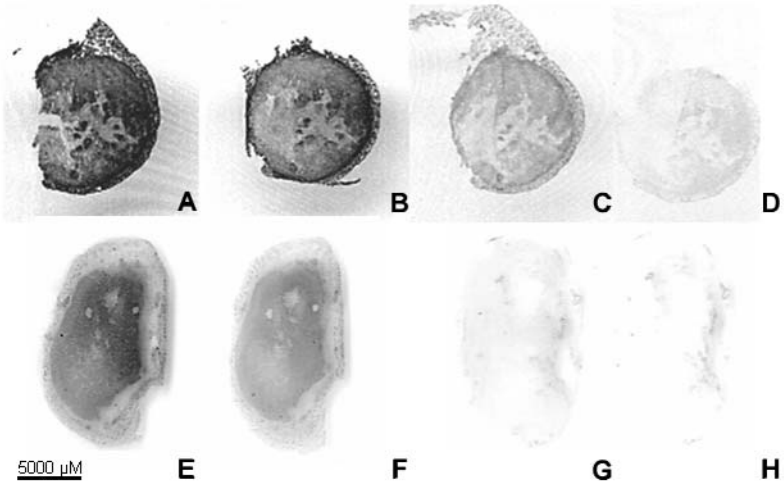

Figure 4. *Immunohistochemistry for NTR in FaDu (A-D) and HT29 (E-H) xenografts for NTR1 (A+E), NTR2 (B+F), NTR3 (C+G) and controls (D+H).*

and xenografts. The expression intensity was normalized to the house-keeping gene 18S rRNA expression.

HT-29 and FaDu cells always expressed higher levels of NTR3 mRNA compared to NTR1 and NTR2. NTR3 expression in the HT-29 cells was about 3.5-fold higher than that of NTR1 and 2.5-fold higher than that of NTR2 (Figure 2). Similar results were observed in FaDu cells with approximately 4-fold higher expression than NTR1 and 2-fold higher expression than that of NTR2 (Figure 2).

NTR expression analysis was further performed in differentiated HT-29 and FaDu cells. RT-PCR analysis revealed a reduced NTR expression in these cells compared to undifferentiated cells (Figure 2). The strongest effect with sodium butyrate to NTR reduced expression could be observed for NTR2 in HT29 cells and NTR1 in FaDu cells (Figure 2).

NTR distribution studies in HT-29 and FaDu corresponding xenografts also revealed a strong expression of NTR3 similar to that found in the tumor cell lines. A high level of NTR1 expression was detectable in these corresponding xenografts compared to the tumor cell lines (Figure 3).

Protein expression of NTR in HT-29 and FaDu cells and corresponding xenografts. To investigate whether the expressed NTR mRNAs were also translated into proteins, immunohistochemical experiments with tumor cells and xenografts were performed with NTR 1, 2 and 3 antibodies. In contrast to mRNA analysis, the corresponding xenografts for FaDu (Figure 4A) and HT-29 (Figure 4E) were strongly immunopositive for NTR1. NTR2 (Figure 4B+F) expression showed a weaker reaction compared with NTR1. The weakest immunopositive signal was detected for NTR3 expression in FaDu (Figure 4C) and HT-29 (Figure 4G) xenografts.

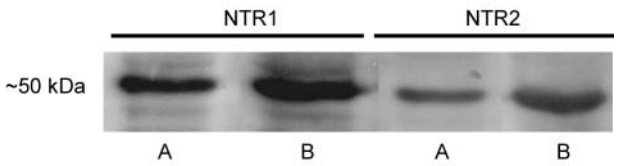

Figure 5. *Immunoblot of 100 ng purified membrane proteins from HT-29 (A) FaDu (B) cells with NTR-1 and NTR-2 antibodies.*

Western blotting analysis of purified membrane proteins from HT-29 and FaDu cells revealed results similar to those obtained for the xenografts. The HT-29 and FaDu cells were strongly immunopositive for NTR1 compared with NTR2 (approximately 50 kDa) (Figure 5). Western blotting experiments with NTR3 revealed no detectable signal in either corresponding xenograft (data not shown).

Discussion

In the present work we studied NTR subtype expression in HT-29 and FaDu tumor cells and in corresponding xenografts showing high endogenous expression. The increasing interest in peptide receptors in cancer research is largely due to the possibility of receptor targeting for tumor diagnosis and therapy. With regard to the NTR system, there is a large body of evidence that NTR1 gene expression is deregulated in human cancer cells derived from a variety of organs. This is well documented in colon, pancreas, prostate and lung cancer (14-17, 22, 23). The

signaling pathway of NTR1 is also well documented (24), leading to its subsequent role in cell proliferation. The biological function of the levocabastine-sensitive NTR2 (25) is still a matter of controversy. NTR3 was also recently shown to be involved in the mediation of growth by stimulation of NT in cancer cell lines (13). We used HT-29 colon adenocarcinoma cells and corresponding xenografts as an accepted tumor model for studying NTR expression. In addition, we showed for the first time that FaDu cells, a squamous cell carcinoma, and the corresponding xenografts also showed high expression of NTR.

For the evaluation of peptide receptor expression for molecular imaging, it is necessary to identify and define *in vitro* potential peptide receptors that may be of interest. Moreover, an examination of receptor expression both in cell lines and in corresponding xenografts is also critical in determining whether they reflect the peptide receptor situation as well. It is also necessary to compare receptor mRNA and receptor protein, because they may differ and since the receptor protein is targeted *in vivo*.

Our mRNA data showed a high expression in the tumor cells in the following order: NTR3>NTR2>NTR1. The FaDu cells revealed a higher receptor expression than the HT-29 cells. Recently, Dal Farra *et al.* (13) studied the expression of NTR in human cancer cells of prostatic, colon and pancreatic origin by RT-PCR and binding experiments. They found that NTR2 was expressed in none of these cells and therefore suggested no involvement in the growth response to NT. In contrast, NTR3 was present in all cell lines and NTR1 was expressed in most, but not all cells. The present results clearly demonstrated NTR2 expression in HT-29 and FaDu cells, originating from colon adenocarcinoma and squamous cell carcinoma, respectively. In contrast to our studies, Dal Farra *et al.* (13) screened seven colon cancer cell lines for NTR expression and suggested that the stimulation of cell proliferation induced by NT was only linked to NTR1 and NTR3, because of the dual expression of NTR1 and NTR3 in most of the colonic and pancreatic cancer cells. Based on our results, we have no evidence for NTR2 as an activator for cell proliferation.

Looking at the corresponding xenografts, NTR3 expression was still high, but decreased when compared to the cultured tumor cells. In contrast, an increase in NTR1 expression in the corresponding xenografts was observed when compared to tumor cells. Recent studies on NTR1 mRNA expression in normal pancreas and pancreatic disease described an overexpression of NTR1 mRNA of pancreatic cancer samples compared to normal controls (23). These studies suggest that increased NTR1 mRNA synthesis is translated into protein in pancreatic cancer cells.

We further studied NTR expression after sodium butyrate treatment. This short-chain fatty acid naturally is present in the human colon and modulates a wide range of cellular processes, including the induction of cell cycle arrest, differentiation and apoptosis in various solid tumor entities including breast, lung and colon cancer (26-29). It has also been suggested that the use of differentiation inducers is an alternative therapeutic approach to conventional tumor therapy. Differentiation results in a slower tumor growth. Cell cycle analysis clearly showed that high concentrations of sodium butyrate (4 mM) induced cell differentiation in both cell lines. We showed for the first time that a squamous cell line (FaDu) could be differentiated by treatment with sodium butyrate. In addition, no apoptosis was measured in our cell lines after four days incubation (data not shown). A different response to various differentiation agents was already demonstrated and this response depend on the malignant potential of individual cell lines (29, 30). A considerable variation among established cell lines was described even within individual specimens (31). We could see a decrease in all three NTR subtypes in both cell lines.

In this context, the strongest effect of sodium butyrate was detected in FaDu cells on NTR1 expression. Only a very weak NTR1 signal could be detected after sodium butyrate incubation for four days. Our data suggest that sodium butyrate-induced differentiation is a stimulus for NTR reduction in human adenocarcinoma and squamous cell carcinoma cell lines.

NTR1 protein expression was high in tumor cells and in corresponding xenografts; NTR2 likewise was also present but to a lower extent. On the other hand, we were unable to detect NTR3 protein with a specific antibody in cultured HT29 or FaDu cells. The NTR3 antibody signal in the corresponding xenografts was very weak. In contrast to our data, the presence of NTR3 in HT-29 cells was demonstrated using another specific antibody (32-34). In those studies, both a 105 kDa and 135 kDa form of NTR3 were identified as two differentially glycosylated forms of the same receptor. These authors also found heterodimerization between NTR1 and NTR3 in HT-29 cells (33).

An explanation of these controversial observations could be related to the binding properties of the specific antibodies employed in the different studies. The antibody used in the present study binds to the N-terminal, extracellular domain of the NTR3 receptor. This site is likely to be sterically hindered by a heterodimerization process. It is assumed that heterodimerization between NTR1 and NTR3 may change the binding properties of the NTR3 antibody.

In conclusion, our results demonstrate NTR expression in two different tumor models, a human colon adenocarcinoma and a human squamous cell carcinoma, both at the mRNA and protein levels. Our results give rise to the assumption that NTR1 is a good target for advanced

cancer diagnosis, *e.g.*, by using positron emission tomography, because of the high protein expression in tumor cells and xenografts. As mentioned above, NTR1 is a G protein-coupled receptor (GPCRs). These receptors trigger intracellular signalling cascades when stimulated by a variety of extracellular mediators. In this line, GPCRs appear to be very interesting target molecules for the development of therapeutically useful drugs. Our results also direct attention to NTR2 as a potential target the diagnosis and therapy of selected tumor entities, however, the physiological consequences of NTR2-mediated signalling still have to be elucidated. In contrast, NTR3 seems to be of minor relevance for these applications.

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