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Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of Monoamine Transporters in Neuroblastoma Cell Lines: Correlations to Meta-iodobenzylguanidine (MIBG) Uptake and Tyrosine Hydroxylase Gene Expression

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Radiolabelled meta-iodobenzylguanidine (MIBG) has been widely used in scintigraphy and targeted radiotherapy in patients with neuroblastoma. Recently, it has been demonstrated that MIBG is incorporated into neuroblastoma cells by the noradrenaline transporter. *In vitro* experiments on SK-N-SH human neuroblastoma cells performed in the present study showed that uptake of MIBG is inhibited by noradrenaline, more so by dopamine and to a lesser extent, by serotonin, indicating that the respective transporters may also contribute to MIBG uptake. However, neither dopamine nor serotonin transporter gene expression was detected. Noradrenaline transporter gene expression was found in 4 of 6 investigated cell lines, which correlated with specific MIBG uptake. Furthermore, an inverse correlation of noradrenaline transporter and tyrosine hydroxylase gene expression, the key regulatory enzyme of catecholamine synthesis, was observed. These data show that MIBG is specifically incorporated only in neuroblastoma cells in which there is noradrenaline transporter gene expression. Furthermore, the catecholamine status in neuroblastoma cells is regulated by a coordinate expression of the key elements of catecholamine synthesis and reuptake systems.

Key words: neuroblastoma, MIBG uptake, noradrenaline transporter, tyrosine hydroxylase, gene expression, catecholamines

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INTRODUCTION

META-IODOBENZYLGUANIDINE (MIBG), a structural analogue of the antihypertensive drug guanethidine [1] is selectively concentrated in neuroectodermal tumour tissue (e.g. neuroblastoma, pheochromocytoma or APUDoma), which have preserved the capacity to recapture extracellular catecholamines and serotonin, which is a feature of neuronal cells. Therefore radiolabelled MIBG has been successfully applied for scintigraphic visualisation of these tumours [2–4] and for targeted radiotherapy [5, 6]. However, encouraging short term therapeutic effects do not result in improved outcome [6] which is possibly due to insufficient MIBG tumour uptake.

The mechanisms of MIBG uptake have been studied in various neuroectodermal cellular systems [2, 7–10], and two different uptake systems have been shown: (i) a saturable, sodium-, temperature-, energy-dependent, imipramine, cocaine and ouabain inhibitable system with high affinity to catecholamines (Uptake 1); and (ii) a nonsaturable, sodium-independent system which is most likely a simple diffusion process. The uptake characteristics of MIBG are similar to the monoamines, noradrenaline, dopamine and serotonin, which are all transported via corresponding specific proteins located in the cellular membrane. Recently, the human noradrenaline, dopamine and serotonin transporter cDNAs were cloned and sequenced [11–13].

Previous investigations showed a correlation between specific MIBG uptake and noradrenaline transporter signal intensities in neuroblastoma cell lines by quantitative polymerase chain reaction analysis (PCR) [14]. Furthermore, HeLa cells transfected with human noradrenaline transporter cDNA, cloned from total SK-N-SH cDNA, showed MIBG uptake with all the characteristics of Uptake 1 [15]. No specific MIBG uptake was observed in HeLa cells transfected with bovine dopamine transporter and rat serotonin transporter cDNA [15]. In the current study, MIBG uptake experiments using SK-N-SH cells showed that dopamine was more inhibitory than noradrenaline. Therefore, the question arose whether the human dopamine transporter is also expressed in different human neuroblastoma cell lines and may contribute to MIBG uptake. Furthermore, serotonin, to a lesser extent, also inhibited MIBG uptake in SK-N-SH cells, and MIBG/serotonin uptake experiments in human platelets and neuroblastoma cells support the possibility of a serotonin transporter mediated MIBG uptake [16].

The aim of this study was to examine noradrenaline, dopamine and serotonin transporter gene expression levels and specific MIBG uptake in human neuroblastoma cell lines, to determine which of these transporters may contribute to MIBG uptake.

Regulation of gene expression for complex transporter proteins, attributed to neuroendocrine functions of neuronal tissues, vary with the level of neuronal differentiation, reflected by several biochemical parameters including catecholamine production [17]. Since a feedback regulation of catecholamine transporter gene expression and catecholamine biosynthesis seemed reasonable, analysis of tyrosine hydroxylase gene expression, the first step enzyme in catecholamine biosynthesis, was included to investigate a possible correlation with noradrenaline transporter gene expression.

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MATERIALS AND METHODS

Chemicals

[^{131}I]MIBG (specific activity: 140 MBq/μmol) and [^{125}I]MIBG (specific activity: 118 MBq/μmol) were purchased from Amersham Buchler (Braunschweig, Germany). Serotonin and noradrenaline were obtained from Sigma (Munich, Germany), phenylalanine, tyrosine, DOPA, dopamine and adrenaline from Serva (Heidelberg, Germany). Oligonucleotides were synthesised and FPLC purified by Pharmacia (Freiburg, Germany). All restriction enzymes were purchased from Boehringer (Mannheim, Germany). Human substantia nigra cDNA was obtained from Clontech (Palo Alto, California, U.S.A.)

Cell culture

The human neuroectodermal and neuroblastoma cell lines SK-N-LO, LS [18], SiMa (available at DSM Braunschweig, Germany), IMR 32, Kelly and SK-N-SH were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, penicillin/streptomycin (100 U/ml) and 10% fetal calf serum in a 5% CO_2, 100% H_2O atmosphere. Prior to [^{131}I/^{125}I]MIBG uptake experiments and RNA isolation, cells were detected by 2 min incubation with trypsin (1 g/l) and washed twice using phosphate-buffered saline (PBS) (Dulbecco, Berlin, Germany). Samples containing 10^7 cells of each cell line were immediately lysed with guanidinium isothiocyanate and 2×10^6 cells were separately used for MIBG uptake experiments in triplicate.

MIBG uptake and uptake inhibition

Experiments were carried out in 15 ml vials containing 2 ml of the cell suspension (10^6 cells/ml). Inhibitors (phenylalanine, tyrosine, DOPA, dopamine, noradrenaline, adrenaline and serotonin) were added to a final concentration of 10^{-5} mol/l, 15 min prior to radiolabelled MIBG incubation. The reaction was started by adding 20 μl containing [^{131}I]MIBG (Amersham Buchler, Braunschweig, Germany) to a final concentration of 10^{-7} mol/l (0.5 μCi/ml). Total radioactivity was counted prior to incubation at 37°C in a gentle shaking waterbath for 2 h. Experiments were performed in triplicate. After incubation, cells were washed twice with PBS. The incorporated radioactivity was counted by a standard gamma counter. Active molar uptake of [^{131}I]MIBG was calculated using total radioactivity, [^{131}I]MIBG concentration and incorporated radioactivity into 2×10^6 cells minus uptake in the presence of a 100-fold excess of dopamine.

RNA isolation and cDNA synthesis

Preparation of total cellular RNA was performed as previously described [19]. 1 μg RNA was converted to cDNA in a solution of 50 mM Tris pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 3 mM dithiothreitol, 0.1% Nonidet P-40, 500 mM dGTP, dATP, dTTP, dCTP each, 150 pM random hexanucleotide primers (Boehringer Mannheim, Germany) and 0.5 U/ml of RAV2-reverse transcriptase (Amersham Buchler, Braunschweig, Germany). The mixture was incubated for 1 h at 37°C, and cDNA equivalent to 200 ng RNA was used for amplification by PCR after termination of cDNA synthesis and denaturation of nucleic acids by boiling for 3 min.

Polymerase chain reaction

PCR was performed in a final volume of 50 μl containing 10 mM Tris pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 200 mM of dGTP, dATP, dTTP, dCTP each, 2 μM of the amplimers and 2.5 U TAQ-DNA-polymerase (Amersham

Buchler, Braunschweig, Germany). To exclude contamination with PCR products, samples to be used for cDNA synthesis or PCR, respectively, were prepared using separate solutions, pipettes and centrifuges. As a negative control, water instead of RNA or the reverse transcriptase, respectively, were examined at fixed time intervals. Reaction conditions for PCR were 96°C for 15 s, 55°C for 30 s and 72°C for 90 s (Thermocycler 60, Biomed, Theres, Germany). After separation of 10 µl of each PCR product by polyacrylamide gel electrophoresis (8% acrylamide, 0.25% bisacrylamide), the DNA was stained with ethidium bromide, and the signal intensities were evaluated using the CS-1 Videoimager (Cybertech, Berlin, Germany). Signal intensities of the various genes of interest were normalised to the signal intensities of the glyceraldehyde-3-phosphate dehydrogenase (GAPD) amplification product.

PCR primers

PCR was carried out in the exponential range of the amplification kinetics of *GAPD*, noradrenaline, serotonin transporter and tyrosine hydroxylase primer pair. We established *GAPDH* (position: 186–543, 358 bp, 25 cycles, sense 5'CGGG AAGCTTGTGATCAATGG3', antisense 5'GGCAGTGAT GGCATGGACTG3'), noradrenaline transporter (NA) (position: 506–799, 294 bp, 34 cycles, sense 5'GCTTCTACT ACAACGTCATCATC3', antisense 5'CGATGACGACGACC ATCAG3'), serotonin transport (SER) (position: 879–1197, 319 bp, cycles, sense 5'CATCTGGAAAGGCGTCAAG3' antisense 5'CGAAACGAAGCTCGTCATG3') and tyrosine hydroxylase (position: 546–640, 95 bp, 35 cycles, sense 5'GTTCGACCCTGACCTGGACT3', antisense 5'TGTACT GGAAGGCGATCTCA3'). Dopamine transporter (DA) primers A–D were used with 40 and 50 cycles; A: (position 890–1031, 142 bp, sense 5'ATAGACGGCATCAGAGCATACC3', antisense 5'ACTTGTTGTAGCTGGAGAAGGC3') B: (position 324–476, 153 bp, sense 5'ACCTGCTCTTCATGGTCATTGC3', antisense 5'CGACATACAGTGAGATGAGG3'(C: (position: 459–769, 311 bp, sense 5'TCATCTCACTGTATGTCGGC3', antisense 5'CAGCACGATGACCAGCACC3') D: (position: 459–653, 195 bp, sense 5'TCATCTCACTGTATGTCGGC3', antisense 5'GTGTGGTCCCAAAAGTGTCG3'). The identities of PCR products were proven by estimating the molecular weights of the amplified material before and after restriction enzyme digests.

Statistics

Spearman rank order-test was applied to validate the correlations between the expression values of noradrenaline transporter gene expression and MIBG uptake (*rs*). Noradrenaline transporter gene expression and tyrosine hydroxylase gene expression signals were normally distributed (bivariate analysis $P=0.95$), therefore Pearsons' linear regression coefficient was used for correlation analysis (*r*).

RESULTS

We investigated [^{125}I]MIBG uptake into SK-N-SH cells in the presence of 10 × and 100 × excess of catecholamines and serotonin. 0.1 µM [^{125}I]MIBG was used, where a preferential specific accumulation of the drug by Uptake-1 has been shown [20–22]. Of the substances tested, dopamine inhibited MIBG uptake most effectively, followed by noradrenaline and, to a lesser extent, serotonin (Figure 1). These data indicate either that dopamine, and possibly serotonin, block noradrenaline transporters, or that respective transporters for dopamine, and possibly serotonin, are expressed on neuroblastoma cells. This question was addressed by gene expression analysis using RT-PCR.

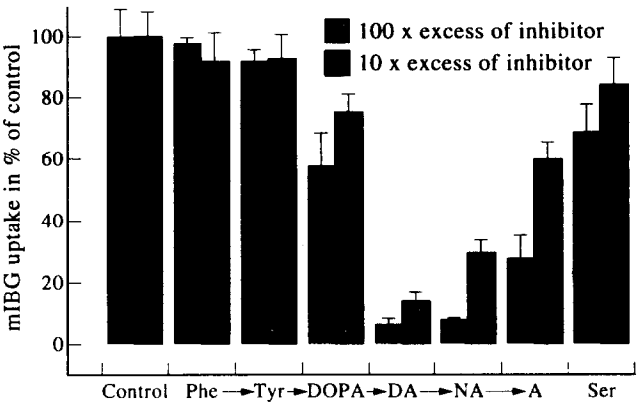

Figure 1. Inhibition of 10^{-7} M [^{125}I]MIBG uptake in SK-N-SH neuroblastoma cells at 37°C (2 h) by catecholamine precursors (phenylalanine (Phe), tyrosine (Tyr), DOPA), catecholamines (dopamine (DA), noradrenaline (NA), adrenaline (A)) and serotonin (Ser) used in 10 and 100-fold excess of MIBG. Bars represent mean ± SD (n=3).

Because dopamine caused the most inhibition of [^{125}I]MIBG uptake, dopamine transporter gene expression was expected in neuroblastoma cells with active MIBG uptake. Therefore, a set of 4 primer pairs A–D was used, which were tested with commercially available human substantia nigra cDNA (Figure 2a). PCR with 40 or 50 cycles using primer pairs A–D did not produce a signal in any of the investigated cell lines,

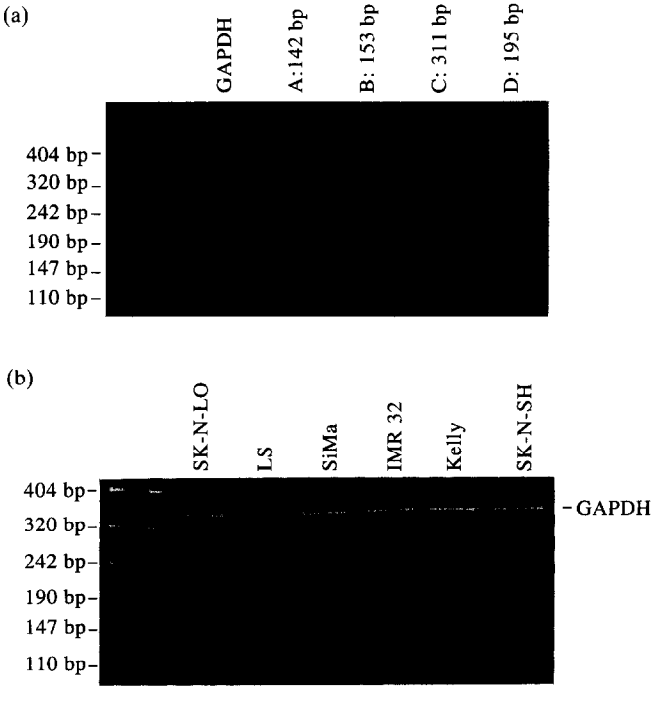

Figure 2. Investigation of dopamine transporter gene expression. (a) Human substantia nigra cDNA served as positive control of dopamine transporter gene expression using a set of 4 different primer pairs. The internal standard *GAPD* and primer pairs A–D were tested, the latter with 40 cycles. (b) cDNA of 6 different neuroblastoma cell lines tested with primer A (40 cycles). Similarly, no signals with primers A–D (using 40 and 50 cycles) were obtained (data not shown).

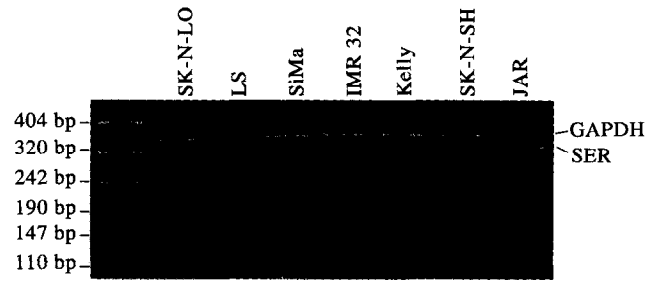

Figure 3. Investigation of serotonin transporter gene expression (SER) in 6 neuroblastoma cell lines. The human trophoblastic cell line JAR was used as a positive control.

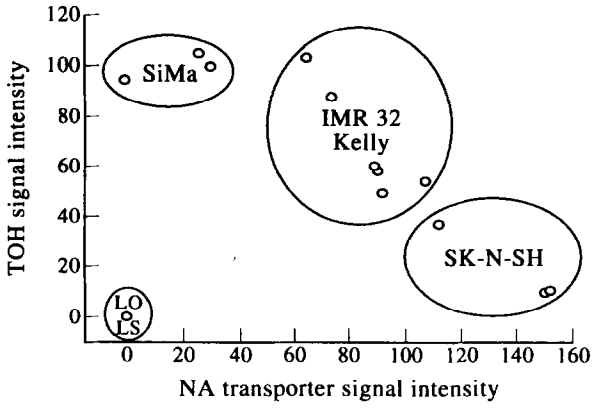

Figure 5. Noradrenaline transporter versus tyrosine hydroxylase signal intensities (in relation to GAPD signal intensity) of 6 neuroblastoma cell lines. Points represent means of three independent experiments. In MIBG positive cell lines (SiMa, IMR 32, Kelly, SK-N-SH) noradrenaline transporter gene expression and tyrosine hydroxylase gene expression were inversely correlated (r=0.920, P<0.0001). LO, SK-N-LO.

which is shown in Figure 2b for primer pair A (40 cycles). cDNA of the human trophoblastic cell line JAR was used for serotonin primer establishment and served as positive control. No serotonin transporter gene expression was found in any of the 6 neuroblastoma cell lines (Figure 3). These data indicate that neither dopamine nor serotonin-transporter are expressed in the neuroblastoma cells lines investigated.

A range of expression levels of the noradrenaline transporter gene was observed in our panel of 6 neuroblastoma cell lines (Figure 4). Parallel to noradrenaline transporter gene expresion analysis, [^{131}I]MIBG uptake in the presence and absence of 10^{-5} mol/l dopamine was measured (Figure 4). SK-N-SH, Kelly, IMR 32 and SiMa were found to have dopamine inhibitable [^{131}I]MIBG uptake, whereas SK-N-LO and LS had no active uptake. A highly significant correlation (rs=1.000, P<0.0001) between noradrenaline transporter gene expression and [^{131}I]MIBG uptake was observed. Tyrosine hydroxylase gene expression was only found in those neuroblastoma cell lines which had the capacity for active [^{131}I]MIBG uptake (Figure 4). Noradrenaline transporter gene expression was inversely correlated with tyrosine hydroxylase expression (r = −0.920, P<0.0001) (Figure 5).

DISCUSSION

Despite some encouraging results in targeted radiotherapy of patients with neuroblastoma using [^{131}I]MIBG [5, 6] therapeutic effects remain poor, possibly due to insufficient [^{131}I]MIBG tumour load [23], which also depends on low cellular MIBG uptake. Transmembranous transport of MIBG has been shown to occur by means of specific monoamine transport systems, generally termed as Uptake 1 [2, 7–10], which is possibly a heterogeneous group of different transporter proteins. The monoamine transporters for dopamine, noradrenaline and serotonin have been cloned and sequenced recently [11–13]. To improve therapeutic efficacy of [^{131}I]MIBG, characterisation of specific MIBG uptake systems in neuroblastoma cells and insights in to their gene expression regulation might be useful in order to improve MIBG uptake by the induction of MIBG transporter gene expression upregulation.

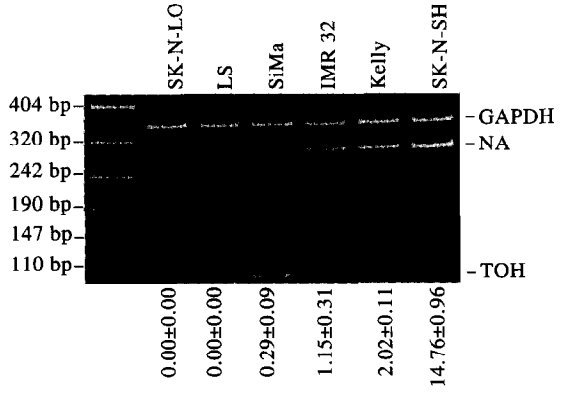

Figure 4. Noradrenaline transporter (NA) and tyrosine hydroxylase (TOH) gene expression in 6 neuroblastoma cell lines versus specific [^{131}I]MIBG uptake (lower figures) (pmol/10^6 cells/2 h; mean±SD, n=3; SK-N-LO, LS cell lines, uptake not detected).

Although dopamine was the most effective MIBG uptake inhibitor (Figure 1), the expected human dopamine transporter gene expression could not be detected in the 6 neuroblastoma cell lines investigated (Figure 2b). A set of 4 different primer pairs was used, which are operational on human substantia nigra cDNA (Figure 2a). Serotonin had some inhibitory effect on MIBG uptake (Figure 1), but, again, no corresponding transporter gene expression was found (Figure 3), although the serotonin transporter was expressed in JAR cells. Noradrenaline transporter gene expression was detected in 4 of 6 cell lines with specific MIBG uptake (Figure 4). In summary, these data indicate that dopamine and serotonin act as competitive inhibitors of noradrenaline transporter-mediated MIBG uptake in neuroblastoma cells, which do not express dopamine and serotonin uptake systems.

Since both dopamine, and to a lesser extent serotonin, are structurally related to noradrenaline, a competitive MIBG uptake inhibition is probable. This interpretation is supported by [^{3}H]noradrenaline (20 nM) uptake inhibition experiments in noradrenaline transporter cDNA transfected HeLa cells in which dopamine ($K_{50\%\ inhibition}$ = 139 nM) was more inhibitory than unlabelled noradrenaline ($K_{50\%\ inhibition}$ = 320 nM) and serotonin, which had only weak effects ($K_{50\%\ inhibition}$>10,000 nM) [11].

While [^{131}I]MIBG accumulation in neuroblastoma cells would appear to be independent of dopamine and serotonin transporters, these may be responsible for radiopharmaceutical uptake in non-targeted tissues. Thrombocytopenia, a major side-effect in [^{131}I]MIBG therapy [23], is thought to be a consequence of serotonin transporter-mediated uptake into thrombocytes or megakaryocytes [16], which can be inhibited by fluvoxamine, a specific serotonin antagonist. However, rat

serotonin transporter cDNA transfected HeLa cells has not shown specific MIBG accumulation [15], therefore, it is possible that another monoamine transporter system (e.g. noradrenaline transporter) is present in megakaryocytes. Negative results of MIBG uptake in dopamine transporter transfected HeLa cells [15] have shown that MIBG is not accumulated by dopamine transporter positive tissues.

In contrast, noradrenaline transporter gene expression has been shown to be present in different MIBG positive tissues [11, 24], and MIBG uptake has occurred in noradrenaline transporter cDNA transfected HeLa cells [15]. The positive correlation of noradrenaline transporter gene expression and MIBG uptake, shown by Mairs and associates [14] and in the present study (Figure 4), suggest this transporter is the major MIBG uptake system. Therefore, attempts to increase its expression seem to be a suitable way to enhance MIBG uptake by neuroblastoma cells. Montaldo and colleagues [25], in cell culture studies using LAN-5 neuroblastoma cells, showed that gamma-interferon, which induces morphological LAN-5 cell differentiation, enhanced both MIBG uptake and noradrenaline transporter gene expression. The intracellular level of noradrenaline has been reported to be decreased in gamma-interferon-treated cells [26], indicating the possibility of a noradrenaline-mediated transporter gene expression regulation.

So far, not much information on noradrenaline transporter gene expression regulation is available. An inverse correlation of noradrenaline transporter gene expression and tyrosine hydroxylase gene expresion was observed (Figure 5). Similarly, some evidence of an inverse correlation of noradrenaline transporter gene expression and noradrenaline uptake was obtained by Szot and associates [27], who found an increased noradrenaline transporter mRNA production in rats treated with desipramine, which also suggests a regulation dependent on intracellular noradrenaline levels. Our own preliminary results showed that incubation of IMR 32 cells with 10^{-5} mol/l retinoic acid resulted in an increased production of catecholamines and decreased noradrenaline transporter gene expression (data not shown). Therefore, pharmacological inhibitors of noradrenaline synthesis should increase noradrenaline transporter gene expression, and a decreased expression is expected in the presence of noradrenaline, which is currently under investigation.

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