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Expression of m2 muscarinic acetylcholine receptor mRNA in primary culture of human prostate stromal cells

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Abstract The aim of this study was to investigate the expression of the muscarinic acetylcholine receptor (mAchR) subtypes mRNA in primary culture of human prostate stromal cells using the reverse transcription polymerase chain reaction (RT-PCR), RNA blotting and in situ hybridization (ISH). Using an explant method, we obtained a primary culture of prostate stromal cells from three patients with benign prostatic hypertrophy. Total RNA was extracted using the acid guanidinium method for cDNA synthesis. First-strand cDNA was then used for PCR with primers designed to amplify the fragments of each mAchR subtypes (m1–m5) cDNA sequence. The m2, m3 and m4 subtype expected bands were detected; in particular m2 transcripts was strongly detected in the stromal cell culture. Each of the PCR products were subcloned into the pGEM-T plasmid vector, sequenced and random primer labeled using ^{32}P. Digoxigenin-labeled cRNA probes were synthesized by in vitro transcription. RNA blotting using a m2 muscarinic receptor cDNA probe revealed a 4.5 kb single transcript. However, m3 and m4 probes did not hybridize. Using in situ hybridization (ISH), m2 receptor mRNA signals were detected in several smooth muscle cells. The staining was predominantly localized to the perinuclear cytoplasm. The m3 and m4 probes did not hybridize. These results suggested that m2 receptor subtype plays a role in smooth muscle activity of the human prostate.

Key words Muscarinic acethylcholine receptor subtypes mRNA expression · Primary culture of human prostate stromal cells · Reverse transcription polymerase chain reaction · RNA blotting In situ hybridization

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Introduction

The existence of mAchR in human prostate has been revealed by Lepor and Kuhar using radioligand receptor binding and autoradiography studies [11]. They demonstrated that these receptors were predominantly localized in the epithelium of the human prostate. Other investigators reported that prostatic secretion in the dog is dramatically enhanced by muscarinic cholinergic agonists [3] and that muscarinic agonists have no effect on the contractile properties of human prostatic adenoma tissues [4, 8]. These findings suggested that mAchR plays a role in neuropharmacology of prostatic secretion. Conversely Yazawa et al. isolated a primary culture of smooth muscle cells existing in the human prostate and characterized mAchR subtype in receptor binding studies [17].

Molecular cloning has identified five genes encoding five structural variants for the mAchR [2, 9]. Four of these gene products (m1, m2, m3 and m4) correspond to the pharmacologically characterized receptors M1, M2, M3 and M4, respectively [6]. Subtypes of mAchR have now been recognized in many tissues, and their localization in the prostate is an important step to the understanding of the function of those receptors and the effects of subtype specific muscarinic drugs.

The purpose of the current study was to investigate the expression of the mAchR subtypes mRNA using the reverse transcription polymerase chain reaction (RT-PCR), RNA blotting and in situ hybridization (ISH) in primary culture of human prostate stromal cells.

Materials and methods

Establishing a primary culture of human prostate stromal cells and epithelial cells

Prostate stromal cell cultures were established using the explant method. Prostate glands were obtained from three patients with benign prostatic hypertrophy undergoing open prostatectomies. The glands were cut into 1 mm cubes with scissors. The tissue

pieces were placed in plastic dishes and submerged in tissue culture medium RPMI 1640 supplemented with 10% fetal bovine serum, 1 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml human transferrin, 5 ng/ml sodium selenite, 100 U/ml penicillin and 100 μg/ml streptomycin. The flask was placed in a humidified cell culture incubator in an atmosphere of 5% CO_2, 95% air at 37 °C to allow outgrowth of prostatic smooth muscle cells. After 10 days, the tissue explants were removed, and the media was replaced with fresh supplemented RPMI 1640 medium. Immunocytochemistry was used to confirm the identity of the muscle cells by staining specifically for human smooth muscle α-actin. The existence of fibroblast cells in the stromal cell culture was confirmed by their characteristic spindle shape and the immunohistochemistry using a monoclonal fibroblast-specific antibody. All experiments were done within two passages. A prostate epithelial cell culture was also established by the explant method using three patients with benign prostatic hypertrophy undergoing open prostatectomies as compared with mAchR subtype expression to stromal cells. To establish epithelial cells, tissues were plated in MCDB 153/RPMI1640 (9:1) containing epidermal growth factor (10 ng/ml), bovine pituitary extract (100 μg/ml), insulin (5 μg/ml), dexamethason (1 μg/ml), bovine serum albumin (100 μg/ml), oleic acid (0.4 μg/ml), heparin (50 μg/ml), cholera toxin (0.1 μg/ml) and gentamicin (50 μg/ml) with 1% fetal calf serum on collagen coated dishes. Epithelial cells showed a typical epithelial morphology and immunohistochemistry demonstrated these to be prostate specific antigen positive. Whole prostate tissues from three patients with benign prostatic hypertrophy undergoing open prostatectomies were also stored for mRNA extraction. Tissues were rapidly frozen immediately after removal and stored −80 °C in a RNase free container.

Reverse transcription polymerase chain reaction

Total RNA was extracted using the acid guanidinium phenol chloroform method [5]. For use in the polymerase chain reaction, total RNA was reverse-transcribed to cDNA by using a DNA amplification reagent kit (GIBCO BRL, USA) The cDNA was synthesized from 5 μg total RNA and 1 μl the first strand cDNA solution was then used for PCR, with each specific primers designed to amplify fragments of each muscarinic receptor cDNA sequence (Table 1). PCR was performed in 50 μl buffer (50 mmol/l KCl; 10 mmol/l Tris-HCl, pH 8.4; 1.5 mmol/l MgCl2 and 200 mg/ml gelatin), with 160 mmol/l of each dNTP, 0.8 mmol/l of each oligonucleotide primer, and 2.5 U of Taq DNA polymerase (Roche Diagnostics, USA). Thirty-five cycles of 30 s (denaturation) at 95 °C, 30 s (annealing) at 62 °C and 1 min (extension) at 72 °C were performed. At the end of the 35 cycles, an additional 10 min extension at 72 °C was added. A 500 bp fragment of the human β–actin gene was amplified as a positive control. To rule out the possibility of amplifying genomic DNA, in some experiments PCR was performed without prior reverse transcription of the RNA. The PCR products were size fractionated by 3% agarose gel electrophoresis. DNA bands were visualized with an ultraviolet transilluminator (Spectoline, Funakoshi, Tokyo Japan).

RNA blotting

Total RNA (20 μg) underwent electrophoresis on a 1% agarose gel containing formaldehyde and was transferred to a nylon membrane (Duralon, Stratagene). This was incubated with prehybridization solution (10 × Denhardt's solution, 5 × SSPE, 2% SDS, 100 μg/ml denatured salmon sperm DNA and 50% formamide) at 42 °C for 2 h. The 288 bp human m2 receptor, 341 bp m3 receptor and 314 bp m4 receptor PCR products were subcloned, sequenced and random primer labeled using ^{32}P [7]. Labeled probe was hybridized at 42 °C for 18 h. The membrane was washed three times at room temperature in 2 × SSC, 0.1%SDS for 10 min, and three times with 0.2 × SSC, 0.1%SDS at 55 °C for 10 min, then subjected to autoradiography.

In situ hybridization (ISH)

Preparation of the cRNA probe

The human muscarinic receptor cRNA probes was prepared by cloning each of the PCR products into pGEM-T plasmid vector (Promega, Madison, Wis.). The resulting PCR products were sequenced. To synthesize anti-sense and sense RNA probe, plasmid DNA that had been linearized with adequate restriction enzyme was transcribed using SP6 or T7 RNA polymerase in the presence of a ribonucleotide mixture (ATP, UTP, GTP and CTP) and digoxigenin-labeled UTP using a commercial kit (Genius 4 RNA labeling kit; Roche Diagnostics, USA) according to the manufacturer's instructions. The incorporation efficiency of digoxygenin-11-dUTP into the probes was quantified using a diluted, labeled, control RNA probe (Genius 4 kit; Roche Diagnostics; USA), and sample spots were dotted on a nylon membrane. The probes were stored at −80 °C.

Preparation of cells

Primary culture cells were grown on Lab-Tek chamber glass slides and fixed with 4% paraformaldehyde in a 0.1 M phosphate buffered saline at 4 °C for 15 min. Then, they were pretreated with HCl to inactivate endogenous alkaline phosphatase and partially digested with proteinase K and acetylated with acetic anhydride.

Hybridization

Each slide was covered with 50 μl of hybridization buffer containing 50% deionidized formamide (Roche Diagnostics, USA), 10% dextran sulfate (Sigma), 1 × Denhardt's solution, 10 mM Tris-HCl (pH 7.6), 600 mM NaCl, 0.25% sodium dodecyl sulphate (Sigma), 1 mM EDTA (Sigma), 200 μg/ml transfer RNA (Sigma), and 200 ng digoxygenin-11-UTP labeled cRNA probe. The slides were covered with a 25 × 25 mm piece of RNase-free parafilm, placed in a humidified chamber, and incubated for 16 h at 50 °C.

Table 1 mAchR subtype specific primers for RT-PCR. The 5′ primer is listed above the 3′ primer in each case

mAchR subtype	Primer 5′-3′	Product (bps)
m1	AAA TAC AGT CAA GAG GCC GAC TAA G CTT GTC CCA GCG GCA AAG CAG C	349
m2	CTA AGC AAA CAT GCA TCA GAA TTG G AAG GTG CAC AAA AGG TGT TAA TGA G	288
m3	ACC CAG CTC CGA GCA GAT GGA C CGG CTG ACT CTA GCT GGA TGG G	341
m4	CAG CCA TTG AGA TTG TGC CTG CC GGT GGC GTT GCA CAG AGC ATA G	314
m5	TCA GAA ATG TGT GGC CTA TAA GTT C TGA CTG GGA CAC ACT TGT CAC AG	304

Washing

Just after hybridization, the parafilm was quickly removed and the slides were rinsed in 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) at 50 °C. They were then immersed in 50% formamide in 2 × SSC at 50 °C for 30 min followed by rinsing in TNE (10 mM Tris-HCl pH 7.6, 500 mM NaCl, 1 mM EDTA) at 37 °C for 10 min. After exposure to 10 μg/ml RNase A (Sigma) in TNE for 30 min, the slides were rinsed in TNE at 37 °C for 10 min, followed by one rinse in 2 × SSC at 50 °C for 20 min, and two rinses in 0.2 × SSC at 50 °C for 20 min.

Detection

Hybridized probes were detected using a nucleic acid detection kit (Roche Diagnostics; USA) according to the manufacturer's instructions. The hybridized probes were visualized using an alkaline phosphatase-labeled digoxigenin antibody and nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as substrate. Color was allowed to develop in the dark at room temperature for 10–16 h in 50 μl buffer containing 22.5 μg of nitro-blue tetrazolium and 8.75 μg 5-bromo-4-chloro-3-indolyl-phosphate. The slides were counterstained for 10–15 s in 0.02% Saflanin-O (Wako Junyaku, Japan). The blue/purple precipitates indicated the presence of each mAchR subtype mRNA.

Controls

Selected tissue sections were hybridized with m2, m3 and m4 sense cRNA probe. To eliminate possible involvement of proteins and DNA in signal formation, some sections were digested with RNase-A (100 μg/ml, 37 °C, 1 h) before the pretreatment step.

All slides were viewed using an Olympus microscope (Tokyo, Japan).

Results

RT-PCR

Total RNA from primary culture of human prostate stromal cells, epithelial cells and whole prostate tissues were used to construct cDNA. PCR products from three prostate stromal cells cDNAs revealed positive amplification of m2, m3 and m4 mAchR subtype genes. The mRNA encoding m2 subtypes were strongly detected compared to other transcripts (Fig. 1). On the other hand, PCR products from three prostate epithelial cells cDNAs revealed positive amplification of m1, m2 and m5 mAchR subtype genes (Fig. 2). All m1–m5 mAchR subtypes were detected in three whole prostate tissues (Fig. 3). No marked variations were observed in three individuals, respectively. PCR products without prior reverse transcription of the RNA, or without prior mRNA, did not reveal positive bands. All PCR products were sequenced using autosequencer to check the identity.

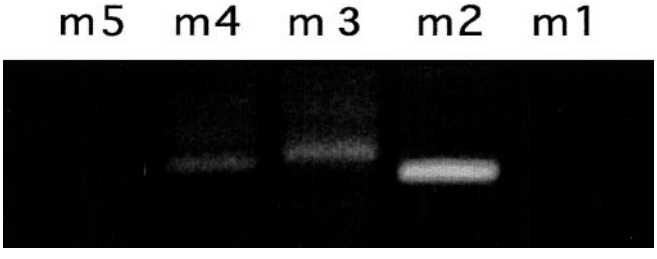

Fig. 1 Detection of m1–m5 mAchR subtype mRNA in stromal cell culture of human prostate by RT-PCR. Three different stromal cell cultures were pooled and used to construct cDNA. Positive amplification of m2, m3 and m4 mAchR subtype genes were detected. The mRNA encoding m2 subtypes was strongly detected compared to other transcripts. Expected band size of m2, m3 and m4 are 288, 341 and 314 bp (base pairs), respectively

RNA blotting

Total RNA from primary culture of human prostate stromal cells (20 μg) was analyzed with ^{32}P labeled m2, m3 and m4 muscarinic receptor probes, respectively. RNA blotting using m2 muscarinic receptor cDNA probe revealed a 4.5 kb single transcript (Fig. 4). However, m3 and m4 probes did not hybridize. Total RNA from epithelial cells (20 μg) was also analyzed with ^{32}P labeled m1, m2 and m5 muscarinic receptor probes, respectively. However, no positive signal was observed.

In situ hybridization (ISH)

Hybridization with m2 muscarinic receptor subtype antisense cRNA probe were detected in stromal cells.

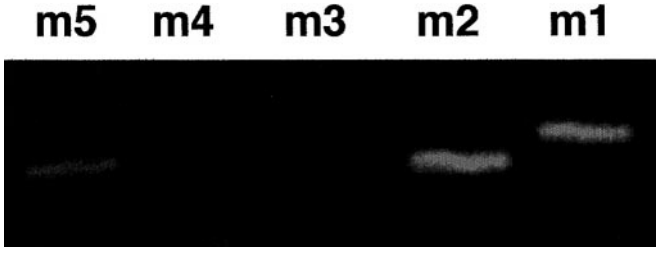

Fig. 2 Detection of m1-m5 mAchR subtype mRNA in epithellal cell culture of human prostate by RT-PCR. Three different epithellal cell cultures were pooled and used to construct cDNA. Positive amplification of m1, m2 and m5 mAchR subtype genes were detected. Expected band size of m1, m2 and m5 are 349, 288 and 304 bp (base pair), respectively

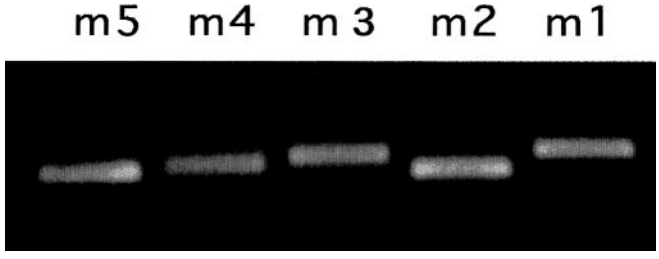

Fig. 3 Detection of m1–m5 mAchR subtype mRNA in human prostate by RT-PCR. Positive amplification of all m1–m5 mAchR subtype genes were detected. Expected band size of m1, m2, m3, m4 and m5 are 349, 288, 341, 314 and 304 bp (base pairs), respectively

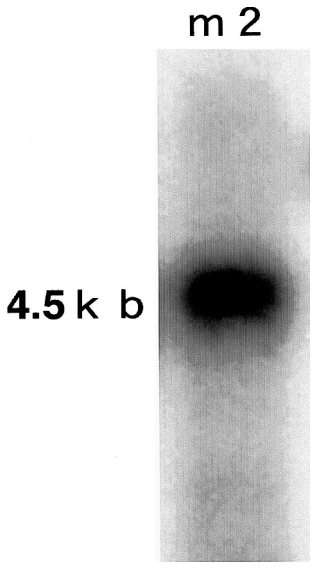

Fig. 4 Expression of m2 mAchR subtype transcript in stromal cell culture from human prostate. The 20 μg total RNA extracted from human prostate stromal cell culture was blotted on nylon membrane and hybridized with a m2 mAchR subtype probe as described in this paper. RNA blotting showed a single transcript of 4.5 kb

The staining were predominantly localized to the perinuclear cytoplasm in several smooth muscle cells (Fig. 5). There was no hybridization with m3 and m4 antisense probes and the m2 sense probe. There was no hybridization with m1, m2 and m5 antisense probes in epithelial cells. The sections which were digested with RNase-A before the post fixation step revealed no staining.

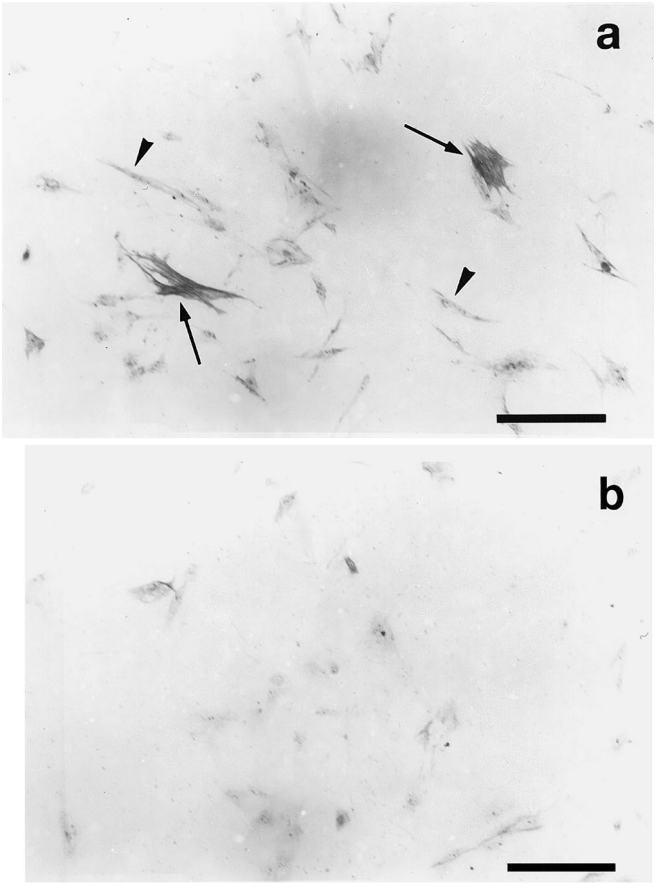

Fig. 5a,b Localization of m2 mAchR subtype mRNA in stromal cell culture of human prostate. Probes were labeled with digoxigenin-11-dUTP. Hybridized probes were visualized using alkaline phosphatase labeled digoxigenin antibody and nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as substrate. Signals obtained using antisense and sense probes are shown (**a**, **b**). Scale bar = 200 μm. Hybridization to m2 mAchR subtype antisense probe was detected in several smooth muscle cells in stromal cell culture from human prostate (*arrow*). Positive staining were predominantly localized to the perinuclear cytoplasm. Note that fibroblasts were negative staining (*arrow head*)

Discussion

Molecular cloning studies have isolated the gene m1, m2, m3 and m4 encoding M1, M2, M3 and M4 muscalinic receptor subtypes, with one additional distinct subtype, the m5 genes [2, 6, 9, 12, 14]. Muscalinic receptors are coupled to G-proteins. It has been suggested that M2 and M4 muscalinic receptors are coupled to inhibition of adenylate cyclase, whereas M1, M3 and M5 receptors are coupled to activation of phospholipase C [13].

Muscarinic cholinoceptor subtypes have been investigated in tissues and cell culture of human and rat prostate by radioligand binding studies and pharmacological contraction studies. [10, 15, 16, 17]. It has been demonstrated that muscalinic receptors in rat prostate are of the M3 subtype [10, 16]. In experiment using a primary culture of smooth muscle cells obtained from patients with benign prostatic hypertrophy, the existence of M2 muscarinic cholinoceptors were demonstrated and those cholinoceptors couple adenylate cyclase inhibition and mediate a decrease in cAMP [17]. On the other hand, Ruggieri and colleagues reported, using both radioligand binding and subtype specific antibodies, that the M1 is the predominant subtype of muscarinic receptor in the human prostate, and that subtype localized to the glandular epithelium. A role of this receptor in cell proliferation has been suggested [12, 15].

In the current study, RNA blot and in situ hybridization showed consistently that the m2 receptor subtype is expressed in primary culture of human prostate stromal cells. Additionally, the results of RT-PCR suggest that m3 and m4 also may be expressed. This observation is consistent with previous report using receptor binding studies in primary culture of human prostate stromal cells [17]. On the other hand, the existence of m1, m2 and m5 were demonstrated in epithelial cell cultures by RT-PCR.

We also investigated the expression of the m1–m5 mAchR subtypes mRNA in the human prostate tissues using RT-PCR. In those experiment, all m1-m5 subtypes were detected in three prostate tissues. The prostate is a heterogeneous tissue composed of epithelial cells,

smooth muscle cells, fibroblasts and connective tissue element. Bartsch and colleagues, using quantitative morphometry, demonstrated that the ratio of stroma to epithelium in the normal prostate is two to one. Benign prostatic hyperplasia is primarily a stromal process, as the ratio of stroma to epithelium is five to one [1]. Although stromal cell culture has the element of smooth muscle cells and fibroblasts, histological observations indicate that stromal cell culture of human prostate contained over 95% smooth muscle cells. The primary culture of stromal cells was chosen to investigate the expression of mAchR subtypes in stromal smooth muscle cells. Then, we clearly demonstrated the existence of m2 receptor mRNA in smooth muscle cells using in situ hybridization. Hybridization with m2 muscarinic receptor subtype antisense cRNA probe were detected and the staining were predominantly localized to the perinuclear cytoplasm in smooth muscle cells. These observation indicates that muscarinic receptor, especially m2 subtype has a role in prostate smooth muscle activities.

In summary, we established primary culture of human prostate stromal cells and detected the strong signal of mRNA encoding m2 mAchR subtypes by RT-PCR. In situ hybridization, hybridization with m2 receptor subtype antisense cRNA probe were detected in smooth muscle cells.

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