Activated Ito Cells of Cirrhotic Liver Express M3 Muscarinic Receptor after Thioacetamide Exposure

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Ito cells or perisinusoidal stellate cells or hepatic fat storing cells are pericytes of normal liver sinusoidal endothelial cells. Activation of Ito cells by chemicals or toxins causes transdifferentiation into the main collagen-producing cells involving in the progression of liver cirrhosis. Quantitative analysis of Ito cell activation by immunohistochemistry has been shown to be useful in predicting the rate of progression of liver fibrosis in some clinical situations. The present study demonstrated that the activated Ito cells in thioacetamide-induced cirrhotic liver, which were immunopositive with alpha smooth muscle actin, expressed M3 muscarinic receptor but not M1, M2, M4 and M5. These findings suggest that the M3 muscarinic receptor might involve in triggering intracellular signalling pathways in activated Ito cell to produce collagen fibers and may be of future interest in hepatic fibrosis therapy.

Keywords: *Ito cells, Thioacetamide, Cirrhosis, Muscarinic receptor*

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Muscarinic receptors are members of the superfamily of G–protein couple receptors (GPCR) binding to acetylcholine (Ach) which acts as a ligand. At present, muscarinic receptor is divided into 5 subtypes; M1-M5. All of them mainly appear in organs containg smooth muscles[1,2]. M1, M3 and M5 receptors are bound to the ligands and activate through calcium. In contrast, the results of stimulation of M2 and M4 muscarinic receptors mediate inhibitory rather than stimulation effect through cAMP[2-5]. The expression of the five subtypes of muscarinc receptors vary considerably in different tissue[6].

M2 receptors in human and animals smooth muscles usually appear more than M3 receptors with the ratio 3-9:1 depending on the species[5-12]. However, the functional studies by pharmacological and biochemistry techniques indicated that M3 receptor causes direct contraction to the smooth muscle cells while M2 receptor results in indirect contraction to the smooth muscle cells. Interestingly, there are reports stating that muscarinic receptors also present at the epithelium in gastrointestinal and urinary tracts as well as white blood cell. However, the function of muscarinic receptors presented is not clearly understood[13,14].

Numerous studies have explored the roles of specific muscarinic receptor subtypes in mediating the diverse physiological actions of acetylcholine[14]. Knowledge about these roles is essential for the development of effective therapeutic approaches aimed at inhibiting or enhancing signaling through specific muscarinic receptors subtypes. However, the task of assigning specific physiological functions to distinct muscarinic receptors subtypes would benefit from knowledge of species distribution of these receptors in the tissue components of an organ. Such information would also reveal to what extent cells express more than one muscarinic receptors subtype[6].

Till now, new concept of "non-neuronal cholinergic system" has been proposed[15]. It proporse

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that some epithelial cells (not only some neurons) can synthesize acetylcholine (Ach) and express muscarinic receptors themselves. Epithelial Ach, therefore, could be bound to the muscarinic receptors expressed on their cell and thereafter mediate locally as an autocrine function or affect the behavior of nearby cells as a paracrine function[14].

Knowledge about expression of muscarinic receptors in the normal and diseased livers is very limit and not be clarified yet. It has been suggested that existence muscarinic receptors on the endothelium of the hepatic sinusoid might be related with the phenomena after cirrhosis[15]. This was the first study to elucidate expression and distribution of muscarinic receptor subtypes in the hepatocyte and activated hepatic stellate cells of the cirrhotic rat liver by immunohistochemical technique.

Material and Method

Twenty one male Wistar rats weighing between 150-200 g were used in the present study they were purchased from the national laboratory animal centre (NLAC, Salaya, Thailand). The Rats were kept in the room maintained at 25°C on a standardized (12-hour) light/dark cycle and ad lubitum. All rats were randomly assigned to the control (no treatment: n = 5) and the treated groups (n = 16). Rats in the experimental group were administered with thioacetamide (TAA) for induction of cirrhosis using intraperitoneal injection (200 mg/kg body weight) for 3 times per week of consecutive 16 weeks. At the end of experimental rats in each group were euthanized by carbondioxide or ether inhalation and killed rapidly by decapitation.

Livers were immediately dissected and removed out through a midline abdominal wall incision[16,17].

Liver specimens were fixed using 4% paraformaldehyde for overnight. Thereafter, each specimen was dehydrated in grade series of ethanol and embedded in paraplast. Serial sections of 5-7 μm in thickness was prepared and placed on mounted poly L-lysine-coated slides. Liver samples were histologically stained with hematoxylin and eosin (H&E) and Sirius red in order to identify the degree of liver fibrosis, The remaining slides were stored at 4°C for subsequent immunohistochemical study.

The sections were deparaffinized in xylene, rehydrated and washed in PBS. The sections were then autoclaved in 10 mM sodium citrate pH 6.0 for 10 min to retrieve antigens and to inactivate endogenous alkaline phosphatase, cooled down at room temperature for at least 15 min, washed in PBS, blocked in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20; pH 8.0), containing either 10% normal goat, fetal calf, or rabbit serum, for 30 min in a moist incubation chamber, incubated overnight (without prior washing) at room temperature with primary antisera dissolved in the blocking solution. Subsequently, the sections were washed 3 times in a mixture of PBS and 0.5 M sodium acetate, incubated for 2 hours at room temperature with the alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) or alkaline phosphstase-conjugated goat anti-rabbit IgG (Dako Inc., Glostrup, Denmark). After incubation, the sections were re-washed once more as described. To reveal antibody binding, the sections were incubated with nitroblue tetrazoliumchloride/5-bromo-4-chloro-3-indolyl phosphate (toludine salt; Dako Inc., Glostrup, Denmask) diluted in 100 mM Tris pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$ at room temperature. After stopping the reaction in double distilled water, the sections were quickly dehydrated through an ascending series of graded ethanols, cleared in xylene and mounted in Entellan (Merck). The specificity of the muscarinic receptors antisera were confirmed by omission of the primary antibody[18].

Results

The present study, demonstrated immuno-histochemically that the activated Ito cells in thioacetamide-induced cirrhotic liver (Fig.1), during week 16 following TAA administration, staining for M3 muscarinic receptor could only be observed in the cytoplasm of the activated Ito cells and of the monolayer cells located at the periphery of each hepatic nodules (Fig. 2). Most of the hepatocytes do not stain for M3 receptors.

Conclusion and Discussion

This is the first study demonstrating that the hepatocytes express M2 and M3 muscarinic receptors. Expression of M2 and M3 muscarinic receptors in normal liver shows zonation in pattern. M2 expresses in the pericentral hepatocytes while M3 subtype shows in the periportal hepatocytes. It has been known that the hepatocytes in pericentral and periportal areas perform different metabolic functions. Glycolysis, glutamine synthesis and lypogenesis occur mainly in the pericentral hepatocytes. Gluconeogenesis, glycogen synthesis, urea synthesis, cholic acid and bilirubin excretion, proteolysis and amino acid degradation present in the periportal hepatocytes[19].

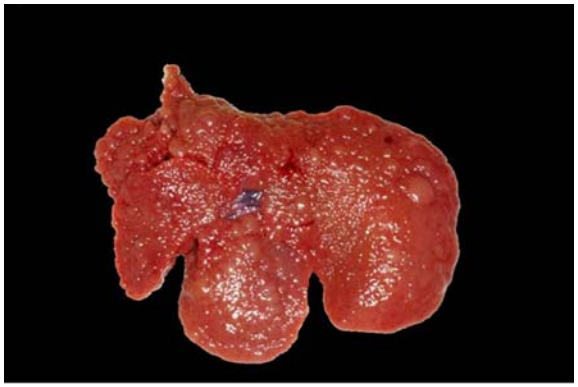

Fig. 1 Photomicrograph showing the liver treated with thioacetamide for 4 months. Note many nodules with varying sizes on the hepatic surface

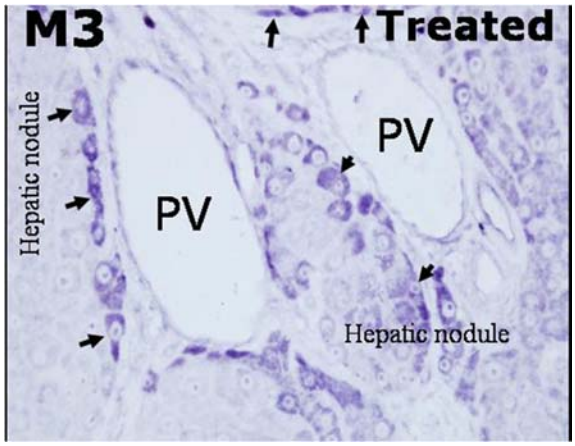

Fig. 2 Expression of M3 muscarinic receptor in the activated Ito cells (arrows). PV=portal vein

From our findings, it is likely that those mentioned metabolic functions, if mediate through Ach, M2 and M3 subtypes of muscarinic receptor could be responsible for the functions of pericentral and periportal hepatocytes, respectively.

The existence of muscarinic receptors is an evidence to support the concept of "non-neuronal cholinergic system'' in the hepatocytes. However, for demonstration such system completely in hepatocytes, scientific evidences for expression of choline acetyltransferase (ChAT) which is an important enzyme for Ach production, needs to be further research.

Liver cirrhosis is characterized by hepatic dysfunction where normal hepatic tissue is replaced with collagen-rich extracellular matrix. Ito cells or perisinusoidal hepatic stellate cells (HSCs) or hepatic fat-storing cells are pericytes of normal liver sinusoidal endothelial cells. Activation of HSCs into myofibroblast-like phenotype by some chemicals or toxins is involved in the progression of liver fibrosis and some other hepatic diseases. The alpha-smooth muscle actin is a well known marker of Ito cells activation[20].

The disappearance of zonation expression of muscarinic receptors is observed after the liver get cirrhotic. This presumes the lost of metabolic functions of different zones of hepatocytes and cause abnormal clinical signs and symptoms of cirrhosis.

The authors, from the present study, could also demonstrate immunochemically for the first time that the activated hepatic stellate cells (HSCs) in thioacetamide-induced cirrhotic liver, which are immunopositive for alpha smooth muscle actin, express M3 muscarinic receptor but not M1, M2, M4 and M5. These finding suggest that M3 muscarinic receptor might be involved in triggering the intracellular signalling pathways in activated HSC leading to the production of collagen fibers. If this assumption appears to be true this mechanism may be of future interest in therapy of hepatic fibrosis.

Knowledge about expression of muscarinic receptors in the normal and cirrhotic livers from this study may provide new insights into future functional physiological and pharmacological studies.

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Potential conflicts of intersent

None.

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การแสดงออกของ M3 Muscarinic Receptor ใน Ito cells ของหนูที่ถูก ชักนำให้เกิดตับแข็งด้วยสารไธโออะเซตาไมต์

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ในภาวะตับแข็ง Ito cells จะถูกกระตุ้นและเปลี่ยนแปลงเป็นเซลล์สร้างเนื้อเยื่อพังผืด เป็นที่ทราบดีอยู่ว่า alpha-smooth muscle actin เป็นตัวบ่งชี้ภาวะการถูกกระตุ้นของ Ito cells การวิเคราะห์ปริมาณการถูกกระตุ้นของ Ito cells โด วิธีอิมมู โนฮีสโตเคมี มีประโยชน์ในการพยากรณ์อัตราความรุนแรงของโรคตับแข็ง การศึกษาในครั้งนี้พบว่า หลังจากที่หนูถูกชักนำให้เกิดภาวะตับแข็งโดยใช้สารไธโออะเซตาไมต์และศึกษาด้วยวิธีอิมมูโนฮีสโตเคมี นั้น activated Ito cells ซึ่งให้ผลบวกต่อ alpha smooth muscle actin มีการแสดงออกของ M3 Muscarinic receptor แต่ไม่พบการแสดงออกของ M1, M2, M4 และ M5 อาจเป็นไปได้ว่า M3 Muscarinic Receptor น่าจะมีส่วนเกี่ยวข้องกับกระบวนการกระตุ้น Ito cells เพื่อให้เกิดการสร้าง collagen fiber ซึ่งข้อมูลดังกล่าวอาจจะเป็นประโยชน์ต่อการผลิตยาตัวใหม่ๆ ที่รักษาโรคตับแข็งต่อไปในอนาคต