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Histological Detection of *mmcp-4* Expressing Cells by *In Situ* RT-PCR Method

Tetsuya TANAKA[1)] , Satoshi ASANO[2)] , Tomomi NIIBAYASHI[1)] , Haruki USUDA[1)] , Koichiro WADA[1)]
[1)]*Department of Pharmacology, Shimane University Faculty of Medicine, Izumo, 693-8501, Japan*
[2)]*Department of Pharmaceutical Sciences, International University of Health and Welfare, Ohtawara, 324-8501, Japan*
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Mast cells are highly heterogenic cells meaning that different genes are expressed in each different population of mast cells. So far, it has been very difficult to detect that which individual mast cell expresses which genes *in situ*. In this study, we amplified the mouse mast cell protease *(mmcp)-4* mRNAs by *in situ* RT-PCR method and successfully visualized the signals of amplicons. It was found that the cells expressing *mmcp-4* mRNA were connective tissue type mast cells but not mucosal type mast cells, in mouse tongue and intestine sections. This is the first report that the cells expressing the mast cell-specific gene were detected on histological specimens by *in situ* RT-PCR method. And it is suggested that this method will be a powerful tool for clarifying the relationships between pathological and physiological changes in diseases and many genes expressed in mast cells.

Key words: *in situ* RT-RCR, mast cells, mouse mast cell protease

INTRODUCTION

Mature mast cells（MCs）are not in the circulating blood. After reaching the target tissues, circulating MC precursors differentiate, maturate, and start to express many enzymes, histamine, chemical mediators and cytokines, and so on. Such many products are accumulated in their intracellular granules. Getting the activation signals, MCs release the contents in their granules to microenvironment in where they exist. Subsequently, some contents, mainly histamine, trigger immunological reaction, such as the allergic reaction, and some contents work for reconstitution of damaged tissues and for other biological functions［1-6］.

It is known that mature MCs are highly heterogenous cells. In other words, one MC population expresses a specific gene set and other MC populations express other sets of genes. Such heterogeneity was seen even in a tissue from same animal because it was thought that gene expression in MCs was regulated by microenvironmental milieu at where they existed［1-3］. Mouse MCs are thought to express as many as 10 secretory granule serine endopeptidases which include eight chymases, called mouse mast cell protease（MMCP）-1, 2, 3, 4, 5, 5' and L, and chymase-2, and two tryptases, called MMCP-6 and 7. Among these endopeptidases, full-length cDNA sequences were determined only for *mmcp-1, 2, 4, 5, 6, 7* and *L* but not for others［2］. And it was also reported that other than MMCP-L are specifically expressed in MCs［2, 7］. Commonly, MCs has been classified into two subtypes, which are connective-tissue-type MCs（CTMCs）expressing *mmcp-4* and *5* specifically, and mucosal-type MCs（MTMCs）expressing *mmcp-1* and *2* specifically［2］. So, we selected *mmcp-2* and *4* for the targets to detect their mRNAs by *in situ* RT-PCR.

For clarifying the function of each gene product in MCs, it is very important to define the genes expressed in each MC population on the histological samples. Although immunohistological or *in situ* hybridization method was used for such purpose［2, 8］, it seems that these methods have not enough sensitivity for all cases. For the detection of the

Corresponding author: Tetsuya Tanaka Ph.D.
Department of Pharmacology, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan
Tel: +81-853-20-2134
Fax: +81-853-20-2130
E-mail: tetsu@med.shimane-u.ac.jp

mRNA on the tissue sample by *in situ* hybridization, at least 10 copies of target mRNAs per cells are generally required [9, 10]. On the other hand, it is reported that only one copy of mRNA is able to visualize by *in situ* RT-PCR method [9]. In this study, we tried to detect the *mmcp-4*, CTMCs specific, and *mmcp-2*, MTMCs specific, expressing cells on the histological samples by *in situ* RT-PCR method.

MATERIALS AND METHODS

Animals

Male and female ICR mice (Japan SLC, Hamamatsu, Japan) were used for the experiment. They were sacrificed by bleeding from dorsal artery under anesthetization. Each organ was used for total RNA extraction or allowed to fix with indicated fixatives for histological preparations, immediately after removing. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Shimane University School of Medicine, and the protocols for this study were reviewed and approved by the University's Committee on the Care and Use of Laboratory Animals.

Extraction of RNA and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from each organ by using Isogen RNA extracting solution (Nippon gene, Osaka, Japan). Two micro grams each of total RNA was reverse transcribed into cDNA by 200 units of Molony murine leukemia virus transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA) using 25pg of poly d $(T)_{12\text{-}18}$ (Amersham Biosciences, Piscataway, NJ) as a primer, for 50 min at 42℃ in 20 μl of Tris-HCl buffer (pH 8.3) containing 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP mixture (TaKaRa, Kusatsu, Japan), 10 mM ditiothreitol and 2 units RNase OUT™ recombinant ribonuclease inhibitor (Invitrogen). Reverse transcriptase (RT) -PCR was proceeded in 25 μl of QIAGEN PCR buffer (Qiagen, Valencia, CA) containing 0.25 μl of cDNA solution, 50 pmol of sense and antisense primers, 400 μM dNTP mixture and 1.25 units *Taq* DNA polymerase (Qiagen). *mmcp-2*, *mmcp-4* and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*, as internal control) mRNA expression was analyzed by RT-PCR using the following primer sets, *mmcp-2*: sense 5'-CCACTAAGAACGGTTCGAAGGAG-3', antisense 5'-GCTGGGATGAACTCAGA-GGTACC-3', *mmcp-4*: sense 5'-CGACACTGGCAAGATGCAGG-3', antisense 5'-ACGCAGGTCAGGCTTTTCAC-3', and *gapdh*: sense 5'-CACCATCTTCCAGGA-GCGAG-3', antisense 5'-CAGTGAGCTTCCCGTTCAGC-3'. The 35 cycles for *mmcps* or 25 cycles for *gapdh* of each PCR consisted of a 1 min denaturing step at 94℃, a 30 sec annealing step at 57℃, and a 1 min extension step at 72℃. PCR products, 687bp for *mmcp-2*, 773bp for *mmcp-4*, and 467bp for *gapdh*, were resolved on 1.5% agarose gels.

Histological specimen preparation and detection of MCs

For alcian blue/ safranin staining, removed tissues were fixed in Carnoy's fixative (ethanol: chloroform: acetic acid = 6:3:1). For toluidine blue staining and *in situ* RT-PCR examination, they were fixed in Streck Tissue Fixative (STF, Streck Laboratories, Omaha, NE). STF included diazoidinyl urea, 2-bromo-2-nitropropane-1, 3-diol, zinc sulfate, sodium citrate [11, 12]. All of tissues after fixation were dehydrated by graded ethanol, infiltrated by xylene and embedded in paraffin under RNase-free condition. Then, for alcian blue/ safranin staining and toluidine blue staining, specimens were made by conventional method. For *in situ* RT-PCR examination, consecutive three tissue sections were placed on each *in situ* PCR glass slide (Perkin Elmer, Branchburg, NJ), with one section used as the positive control, one as the negative control, and one as the sample for detection.

To detect MCs, sections were stained by toluidine blue or double stained by alcian blue/ safranin as reported before [8, 13, 14].

In situ RT-PCR examination

Detection of *mmcp-4* and *mmcp-2* mRNA was basically carried out using *in situ* RT-PCR procedure as previous reported [15-19]. *In situ* RT-PCR method consists of 5 steps. These are proteolysis step allowing to increase the enzyme permeability, DNA digestion step to remove genomic DNA, reverse transcription step to make first strand cDNA, PCR step to amplify the cDNA, and detection step

to visualize the amplicons. Until mRNAs were converted to cDNA, the specimens were handled under RNase-free condition. Although two-step PCR procedure was commonly used in the previous reports, we modified it to three-step PCR procedure in this study.

Before proteolysis step, the specimens were deparaffinized, hydrolyzed and equilibrated by 0.01 M phosphate buffered saline (PBS, pH 7.4). Then, the sections were treated with 10 μg/ml proteinase K (Sigma Aldorich, St Louis, MO) in PBS at room temperature. Treating time was optimized for each tissue (from 30 min to 2 h). After rinsing with diethyl pirocarbonate (Wako pure chemical, Osaka) -treated water (DEPC water), proteinase K was heat inactivated at 95℃ for 2 min using a GeneAmp *In Situ* PCR System 1000 (Perkin Elmer). The specimens were post-fixed by immersing the slide in 4% paraformaldehyde for 15 min and washed by PBS.

For removing genomic DNA, 50 μl each of DNase I (Roche, Mannheim, Germany) solution (1 U/μl DNase I in DNase digestion buffer containing 0.1 M NaOAc and 5 mM $MgSO_4$, pH 5.5) was applied onto the sections for sample and negative control. On the other hand, 50 μl of DNase digestion solution without DNase I was applied onto the section for positive control on the same slide glass. The each section with buffer was covered with AmpliCover disc (Perkin Elmer) avoiding to contain the air bubbles and allowed to fix the disc onto slide glass with AmpliCover clip (Perkin Elmer) using an Assembly tool (Perkin Elmer). The slide glass was treated at 37℃ for 16 h and heated at 94℃ for 5 min using a GeneAmp System. Then, the clips and discs were removed from slide glass using Disassembly tool (Perkin Elmer). And the slide glass was washed with DEPC water.

For synthesizing first strand cDNA, 50 μl each of RT solution containing 200 U of M-MLV RT (Invitorogen), 1 mM dNTP mixture, 1 μM *mmcp-4* or *mmcp-2* antisense primer (as above), 10 mM DTT (Sigma), 4 units RNase OUT™ recombinant rebonuclease inhibitor (Invitrogen) and 1x 1st strand buffer (Invitrogen) was applied onto the sections for sample and positive control. Buffer without M-MLT RT was used as a negative control. Buffer and sections were covered with disc and clip as above and the slide was incubated at 42℃ for 50 min, then 94℃ for 5 min using GeneAmp system. After removing the clips and discs from the slide, the sections were washed with DEPC water.

PCR amplification was performed using the hot-start procedure using Assembly tool and GeneAmp system according to the manufacture's instruction (Perkin Elmer). PCR solution was consisted of 0.8 μM each *mmcp-4* or *mmcp-2* sense and antisense primer, 10 U *taq* polymerase (Qiagen), 0.2 mM dNTP mixture (TaKaRa), 10 μM digoxigenin-11-2'-dUTP (Roche) and 1x Qiagen PCR buffer. After covering the buffer and section with disc and clip, slide were incubated first at 94℃ for 5 min (initial denaturation), followed by 35 cycles at 94℃ for 1 min, 57℃ for 0.5 min and 72℃ for 1 min for denaturing, annealing and extension, respectively.

After PCR amplification, slide was washed in 0.1 M Tris-buffered saline, pH 9.5, containing 0.15 M NaCl and 50 mM $MgCl_2$ (washing buffer), and soaked in 0.1 x SSC containing 0.4% bovine serum albumin (Sigma) at 45 for 30 min to block nonspecific binding activity (stringent wash). To visualize the amplicons, immunohistochemical signal detection was carried out using mouse anti-digoxigenin monoclonal antibody Fab fragments conjugated to alkaline phosphatase (Roche). 50 μl each of 1:200 diluted antibody in 0.1 M Tris-buffered saline, pH 9.5 containing 0.15 M NaCl was applied onto the section, and the slide was incubated at room temperature for 1 h in a humidified chamber. After washing with wash buffer, signals visualized by nitroblue tetrazolium chloride (NBT, Sigma) and bromochloroindoxyl phosphate (BCIP, Sigma). The specimen was counter-stained with fast green F (Sigma) or picric acid (Sigma), if needed, and mounted with glycerol for microscopic observation.

RESULTS AND DISCUSSION

This is the first report that the cells expressing the mast cell-specific gene, *mmcp-4*, were detected on histological specimens by *in situ* RT-PCR method. And it is suggested that this method will be a powerful tool for clarifying the relationships between pathological and physiological changes in diseases and many genes expressed in mast cells.

It is known that mature MCs are highly heterogeneous cells. In other words, one MC population expresses one specific gene set and other MC populations express other sets of genes. Such heterogeneity was seen even in one tissue from same animal because it was thought that gene expression in MCs was regulated by microenvironmental milieu at where they existed [1-3]. Moreover, it has been known that the gene expression pattern is different from one mouse strain to others [2 and our personal communication]. Then, to choose the tissues used for this study, we investigated the expression pattern of *mmcp-2* and *4* in ICR mouse tissues by RT-PCR. As shown in Table 1, *mmcp-2* mRNAs were detected only in intestine and stomach. Among the organs used in this experiment, gastrointestinal tracts and lung contain the mucosal tissue. So, it was expected that *mmcp-2* mRNAs were detected also in lung,

Table 1. Expression pattern of *mmcp-2* and *mmcp-4* mRNA in ICR mouse tissues

Tissues	*mmcp-2*	*mmcp-4*
tongue	--	++
liver	--	--
skin	--	++
lung	--	+
intestine	+	+
heart	--	++
kidney	--	+
spleen	--	+
stomach	+	++
brain	--	+
muscle	--	++

--; not expressed, +; expressed, ++; strongly expressed

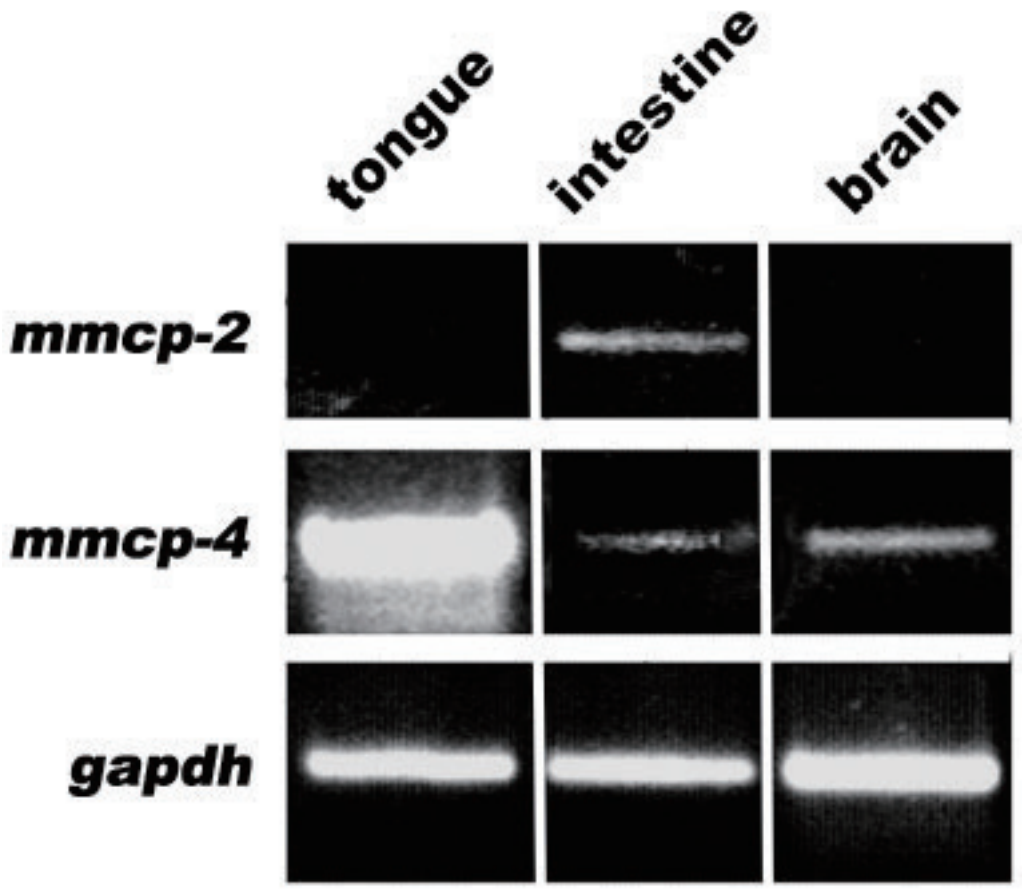

Fig. 1. Expression of mRNA for *mmcp-2*, *mmcp-4* and *gapdh* in tissues from ICR mouse

but not in this experiment. In human lung, it was reported that MCs containing only tryptase (MCT, it was thought to be MTMC in human) were detected and the number of MCT was increased in asthmatic diseases [21]. However, mRNA for *mmcp-2* was not detected in ICR mouse lung thought to containing MTMCs. This result may be come from one of the following reasons. At first, on the grounds that human homologues of MMCP-2 has been never reported [2] and MMCP-2 is chymase but not tryptase, mouse MTMC do not have identical characters to human MCT. Secondly, the lung tissues used in this experiment were obtained from normal mouse, not from asthmatic mouse that had increased number of MTMCs. Finally, although we did not investigate the *mmcp-2* expression pattern in other mouse strains, it may be a specific feature of lung MCs from ICR mouse. On the other hand, *mmcp-4* mRNA was detected in all tissues other than liver in different levels (Table 1). So far, there is no report that indicate the existence of MCs in liver of any animals and are many reports that show the existence of MCs in other organs used in this experiment, excepting brain, by metachromatic staining, immunohistochemical method or RT-PCR, corresponding to our present results. There is a very few report that shows MCs in brain. For example, Orr and Pace reported that the number of MCs in a whole brain of WBB6F1 mouse was deduced up to 100 by counting the number of toluidine blue-stained MCs on 7 μm section (x 10 sections) [22]. Although they did not show the picture of stained sections, it is thought that the number of MCs on one section was from zero to a very few. Based on our RT-PCR results (Table 1 and Fig. 1) and previous reports, we decided to select the following three organs used for detecting the MCs expressing *mmcp-2* and/or *4* by *in situ* RT-PCR method. 1) Tongue that has MCs expressing *mmcp-4* but not *mmcp-2*. 2) Intestine that has MCs expressing *mmcp-2* and/or *4*. 3) Brain that has MCs expressing *mmcp-4* but hard to detect the MCs on histological specimen by previous methods.

The cells expressing *mmcp-4* mRNA on tongue, intestine and brain section were tried to detect by *in situ* RT-PCR method. On the negative control section, no signal was detected (data not shown) and on positive control section, many signals could be seen in almost all cells and the signals were seemed to be restricted in the nuclei (Fig. 2, G, H and I). The signals on positive control sections came from amplified genomic DNAs in nuclei, because positive control sections were omitted to pretreat with DNase I. Judging from the results obtained from negative and positive control sections, the optimizations of treating time and concentration of agents in each step were successfully done for *mmcp-4* detection. It was thought that tongue contains only CTMCs and intestine contains both CTMCs and MTMCs [2]. In our present study, transcripts for *mmcp-4* were detected in the cells among tongue muscle fibers (arrowheads in Fig. 2, D) at where MCs were observed by metachromatic staining (Fig. 2, A). And on the intestine section, signals were detected only in the muscularis mucosa but not in other portion (arrowheads in Fig. 2, E). From the results of metachromatic staining, MCs in intestine sections were observed at two portions, one was in the muscularis mucosa (arrowheads in Fig. 2, B), and another was around the junction between villi and submucosa (arrowheads in Fig. 2, C), thought to be CTMCs and MTMCs, respectively. Therefore, it was suggested that *mmcp-4* expressing cells were only in the muscularis mucosa, in other words, only CTMTs were expressing *mmcp-4* messages. We could not detect *mmcp-4* messages on brain sections in present experiment (Fig. 2, F), although the PCR products were detected by RT-PCR method using total RNA from brain (Fig. 1). It may be came from the reason that one brain section contains a very few number of MCs or many single sections contain no MC [22].

We tried to detect the messages of *mmcp-2* by *in situ* RT-PCR method. However, we could not observe the clear signals for *mmcp-2*. In the case for detecting the *mmcp-4* signals on tongue sections, the number of cells that have positive signals was lower than that of cells metachromatically stained with toluidine blue (data not shown). Therefore, it is thought that not all MCs constitutively transcribe the *mmcp-2* and *4* or the speed of their mRNA turn over is very quick, once the translated products are plentifully accumulated in the secretion granules. But, it seems that major reason for fail-

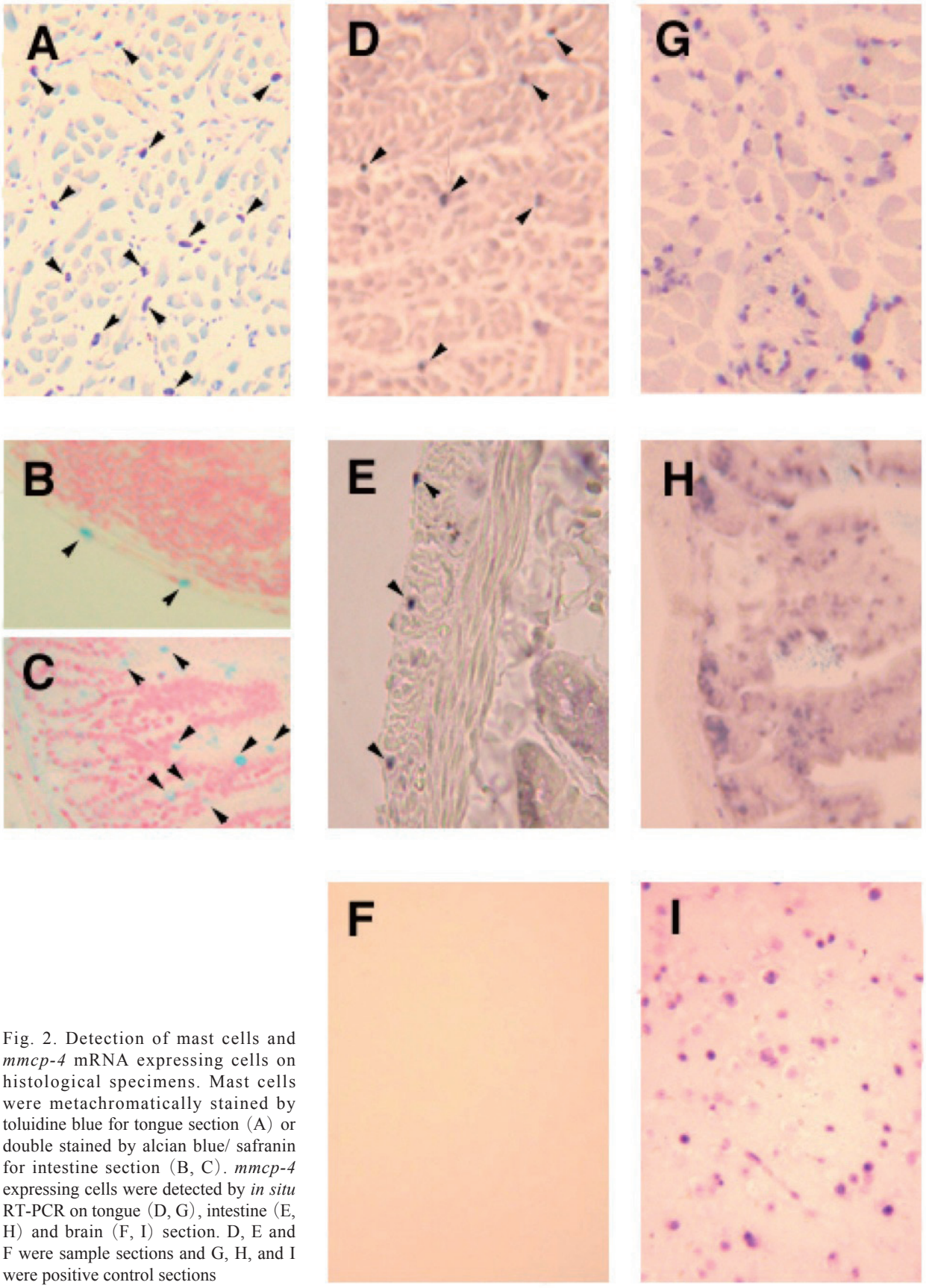

Fig. 2. Detection of mast cells and *mmcp-4* mRNA expressing cells on histological specimens. Mast cells were metachromatically stained by toluidine blue for tongue section (A) or double stained by alcian blue/ safranin for intestine section (B, C). *mmcp-4* expressing cells were detected by *in situ* RT-PCR on tongue (D, G), intestine (E, H) and brain (F, I) section. D, E and F were sample sections and G, H, and I were positive control sections

ing results come from that the treating conditions in one or more steps in *in situ* RT-PCR method did not fully optimized to detect the message of *mmcp-2*. Because, mRNAs for *mmcp-2* was actually exist indicated by RT-PCR using total RNA from intestine (Fig. 1), and we could not obtain the sufficient results even in the positive and negative control sections used for *mmcp-2* detection (data not shown).

In summary, we detected the cells expressing mouse MC-specific gene, *mmcp-4*, on histological samples and differentiated them from other MCs by *in situ* RT-PCR. And we suggest that the method will be useful to visualize a single cell that express a specific gene on section, after optimizing the treating conditions. Therefore, it will be a powerful tool for clarifying the relationships between pathological and physiological changes in diseases and a specific gene.

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