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Expression and localization of TRPV1 in human nasal mucosa*

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SUMMARY

Capsaicin is the pungent principle in chili peppers and previous studies reported that topical application of capsaicin to patients with allergic and non-allergic rhinitis produced significant and long-lasting relief of symptoms. The capsaicin receptor (TRPV1, VR1) is a nociceptive transducer and the existence of TRPV1 in non-neuronal cells as well as neuronal cells has been reported. In order to clarify the role of TRPV1 on the upper airway, we examined the localization and the expression of TRPV1 in human nasal mucosa.

Surgically obtained human nasal specimens were processed for immunohistochemistry with commercial anti-TRPV1 antibody. We also performed immunofluorescence with anti-TRPV1 antibody and anti-neurofilament antibody or anti-CD31 antibody. Epithelial cells and vascular endothelial cells were cultured from nasal turbinates, respectively. For RT-PCR analysis, total RNA was isolated, and then RT-PCR was performed.

Immunohistochemical studies revealed that TRPV1 positive cells were found on epithelial cells, vascular endothelial cells, submucosal glands and nerves in human nasal mucosa. By RT-PCR analysis, the mRNA expression of TRPV1 was confirmed in human nasal mucosa.

These results suggest that capsaicin can directly influence the epithelial secretory and various functions via TRPV1 as well as the activation of the sensory neurons.

Key words: capsaicin, human nasal mucosa, TRPV1, vanilloid receptor, upper airway

INTRODUCTION

Capsaicin (8-methyl-n-vanillyl-6-nomamide) is the pungent principle in chili peppers and activates primary afferent neurons, notably the polymodal nociceptors [1,2]. The capsaicin receptor, previously named the vanilloid receptor subtype 1 (VR1), is a nociceptive transducer activated by capsaicin, heat and protons and it was cloned from rat dorsal root ganglia in 1997 [3]. VR1 is a cell membrane-bound ligand-gated calcium channel with six transmembrane domains, and belongs to the transient receptor potential (TRP) cation channel superfamily [3,4]. Then, VR1 was renamed into TRPV1 and this new nomenclature has been used.

Several authors reported that repetitive challenges of capsaicin in the nasal cavity of the patients with allergic rhinitis [5] and chronic non-allergic rhinitis [6] resulted in statistically significant and long-lasting relief of symptoms. Although TRPV1 expression has previously been localized to neurons that convey nociceptive transmission, TRPV1-immunoreactivity has recently been identified in non-neuronal cells [7-14] as well as neuronal cells. However, the localization and the expression of TRPV1 in human nasal mucosa has not been well elucidated. In order to clarify the role of TRPV1 on the upper airway, we examined the localization and the expression of TRPV1 in human nasal mucosa using immunohistochemical techniques and RT-PCR.

MATERIALS AND METHODS

Tissue preparation

Human turbinates were obtained by turbinectomy from 6 patients with nasal obstruction refractory to medical therapy. Informed consent was obtained from all patients and this study was approved by the ethics committee, Sapporo Medical University. Donors ranged in age from 16 to 49 years: four male and two female, and all were nonsmokers. All medications, including antibiotics, were prohibited for at least 3 weeks prior to the study. Three patients were diagnosed with allergic rhinitis by history and CAP-RAST. The nasal specimens were dissected from the cartilage, and (1) immediately frozen in liquid nitrogen and stored at –70°C for RNA extraction, (2) fixed in 10% formalin for immunohistochemistry and (3) placed into cold transfer medium (Ham's F-12 medium) for epithelial cell culture.

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Antibodies

For immunohistochemistry, a polyclonal antibody against TRPV1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1:50 dilution. To identify the subsets of cells expressing TRPV1, the following monoclonal antibodies were used: anti-neurofilament (DAKO Corporation, Carpinteria, CA, USA) for neurofilament and anti-CD31 (DAKO) for vascular endothelial cells.

Immunohistochemistry and immunofluorescence

Deparaffinized sections were initially incubated with 3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity, washed two times in PBS. After microwave treatment (10 minutes at 500 Watt in citrate buffer), the sections were processed in blocking solution (Antibody Diluent with Background Reducing Components, DAKO) for 30 min. Then, the sections were incubated with anti-TRPV1 antibody overnight at 4°C, washed, and incubated for 30 minutes with EnVision+, Peroxidase, Rabbit (DAKO). A further washing in PBS was followed by developing with DAB (DAKO) as a chromogen for signal visualization. The slides were counterstained with Mayer's hematoxylin and coverslipped using mounting medium. Negative controls were obtained by the rabbit universal negative control (DAKO).

In order to clarify the expression of TRPV1 in neurons and vascular endothelial cells, we performed immunofluorescence for TRPV1 and neurofilament or CD31. Following deparaffinization and microwave treatment, the sections were incubated overnight at 4°C with a combination of rabbit polyclonal anti-human TRPV1 antibody (diluted 1:100) and mouse monoclonal anti-human neurofilament antibody (2F11 clone, DAKO) or anti-human CD31 antibody (JC/70A clone, DAKO). Sections were washed in PBS and incubated for 30 min with Alexa Fluor 594-labelled goat anti-mouse IgG (diluted 1:50; Molecular Probes, OR, USA) and Alexa Fluor 488-labelled goat anti-rabbit IgG (diluted 1:100; Molecular Probes). Sections were mounted with SlowFade antifade kits (Molecular Probes) and examined under an Olympus BX51 microscope equipped with a DP70 CCD camera (Olympus Optical Co., Tokyo, Japan). All images were processed with DP Controller and DP Manager software (Olympus Optical Co.) for image analysis. With this method, TRPV1 expressing cells were green, neurofilament or CD31 positive cells were stained red, and the combined signals were visualized as yellow.

Epithelial Cell culture

Human nasal epithelial cells (HNECs) were isolated from human nasal mucosa according to the previously described protocol [15]. Nasal specimens were rinsed 2-3 times with Ham's F-12 medium (Sigma, St Louis, MO, USA) supplemented with antibiotics and incubated in 0.1% protease type XIV in Ham's F-12 medium for 16 hours at 4°C. After incubation, 10% fetal bovine serum was added to neutralize protease activity and epithelial cells were detached by gentle agitation. Cell suspensions were filtered through a 60-mesh cell dissociation sieve (Sigma) and centrifuged at 500 g for 10 min at room temperature. The cell pellet was then resuspended in hormonally defined Ham's F-12 culture medium (Ham's HD) with the following reagents: 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml amphotericin B, 150 μg/ml glutamine, 5 μg/ml transferrin, 5 μg/ml insulin, 25 ng/ml epidermal growth factor, 15 μg/ml endothelial cell growth factor supplement, 200 pM triiodothyronine, and 100 nM hydrocortisone. Cell suspensions (105 cells/well) were plated onto collagen type-I-coated 6-well culture plates (Sumitomo Bakelite Co. Ltd., Osaka, Japan) in Ham's HD medium and cultured in a 5% carbon dioxide humidified atmosphere at 37°C. The culture medium was changed at day 1 and every two days thereafter. Monolayer cell confluence was achieved after 6-8 days of culture. Morphologic observations using a phase contrast microscope showed the HNECs consisted primarily of epithelial cells. More than 95% of the NHECs showed positive reactions for anti-human cytokeratin Ab (DAKO, Denmark). HNECs grown to 80% confluency were used for RT-PCR analysis.

Human mucosal microvascular endothelial cell culture

Human mucosal microvascular endothelial cells (HMMECs) were isolated from human nasal turbinate by a modified methods of Davidson et al. [16,17]. Surgically obtained nasal mucosa was washed several times with antibiotics contained Ham's F-12 medium and then the epithelial layer and the periosteum were removed with scissors. The mucosal tissues were cut into 4 mm square sections with a scalpel and incubated for 70 minutes at 37°C in trypsin solution in DPBS (Dulbecco's phosphate buffered saline: Gibco, Invitrogen Co., Carlsbad, CA) containing 0.5% collagenase (type II) and 1% EDTA. After three washes with DPBS, the sections were placed into MCDB 131 (Gibco) containing 20% fetal bovine serum (FBS) in a culture dish. Each section was pressed with mucosa elevator outwards from its centre to push out the endothelial cells. The cell suspensions were centrifuged at 500 g for 10 min at room temperature and the pellet was then resuspended in MCDB 131 with 20% FBS and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml amphotericin B). The cells were plated onto a collagen type-I-coated 6-well culture plates (Sumitomo Bakelite Co. Ltd.) and cultured at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. After a 70 minutes incubation, the medium was removed and the dish was washed with DPBS and the cells were cultured in MCDB 131 containing 10% FBS and 75 μg/ml ECGS (endothelial cell growth supplement: Collaborative Research Inc., Bedford, MA). The culture medium was changed at day 1 and every other day thereafter. As in the case of the epithelial cell culture, HMMECs were used for RT-PCR analysis.

RNA extraction and RT-PCR analysis

Total RNA from inferior turbinate was isolated by guanidinium thiocyanate-phenol- chloroform extraction. RT-PCR was carried out using a commercial One Step RNA PCR Kit (Takara Biomedicals, Tokyo, Japan). RNA (50 ng) was reverse-transcribed into cDNA by incubating with 5U AMV Reverse Transcriptase XL at 50°C for 30 minutes. After denaturation at 94°C for 2 minutes, cDNAs were amplified by 40 cycles of PCR (94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 90 seconds) with 5U of AMV-Optimized Taq DNA polymerase to amplify genes for TRPV1. The TRPV1 primers used in PCR spanned a PCR product of 676 bp and the sequences were as follows: sense, 5'-CTCCTACAACAGCCTGTAC-3'; and anti-sense, 5'-AAGGCCCAGTGTTGACAGTG-3'(18). PCR products were analyzed by electrophoresis through a 1.5% agarose gel and visualized with ethidium bromide.

RESULTS

Immunohistochemistry and Immunofluorescence

TRPV1 distribution in nasal mucosa was examined using a rabbit polyclonal antibody against a peptide corresponding to amino acid 1-150 of the N-terminal of human TRPV1. In 6 of 6 patients, immunoreactivity for TRPV1 was detected in epithelial cells, vascular endothelial cells, submucosal glands and nerves (Figure 1). Goblet cells and mucous cells of the submucosal glands expressed higher levels of TRPV1 immunoreactivity in the nasal mucosa (Figure 2). Absence of labelling with the negative control confirmed specificity of the staining. The double immunofluorescence staining shows colocalized expression of TRPV1 and neurofilament or CD31 (Figures 3 and 4). No difference was seen in immunoreactivity between the allergic and the non-allergic patients.

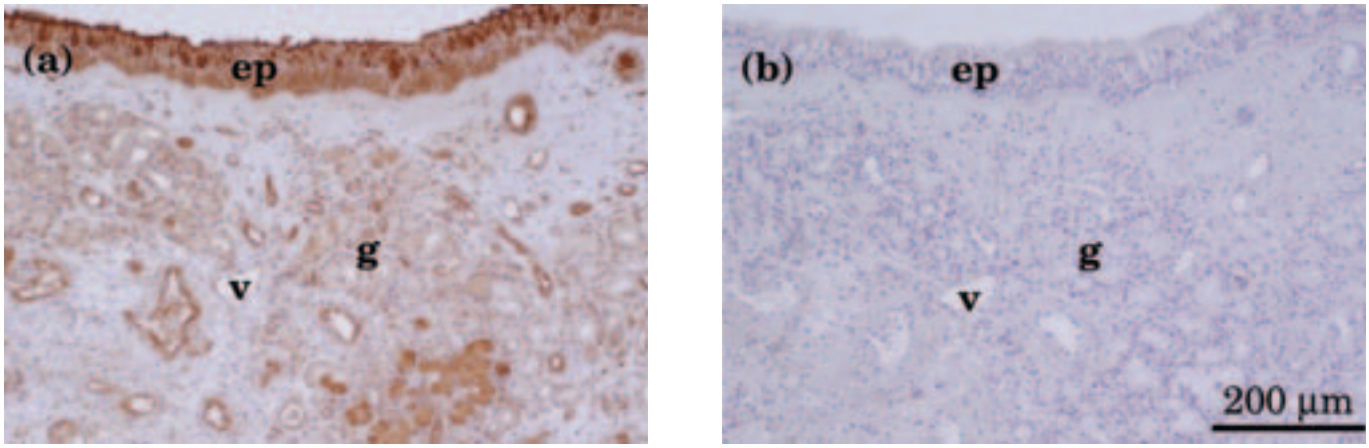

Figure 1. Immunohistochemical staining for TRPV1 in human nasal mucosa. Inferior turbinates were stained with anti-human TRPV1 antibody (a). Staining was observed mainly on epithelium (ep), submucosal glands (g) and vascular endothelium. No activity was seen in the negative control (b). v : vessels.

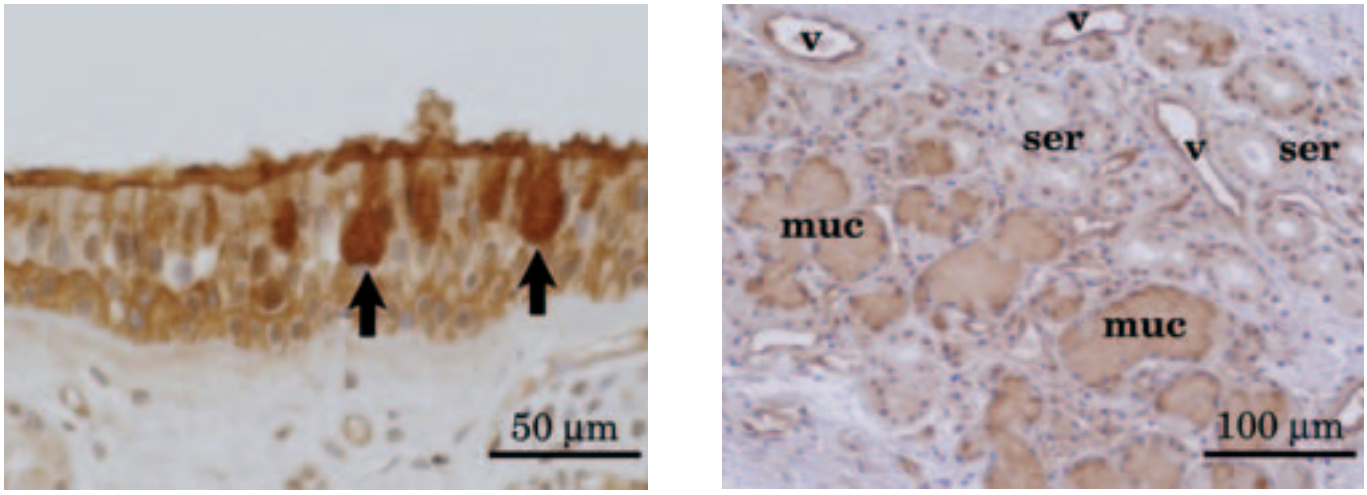

Figure 2. Goblet cells (arrows) and submucosal gland mucous cells (muc) expressed higher levels of TRPV1 immunoreactivity in the nasal mucosa. ser : serous cells.

RT-PCR

Figure 5 shows the results of RT-PCR using total RNA extracted from human nasal mucosa, cultured human nasal epithelial cells and cultured vascular endothelial cells which revealed the expression of TRPV1 mRNA (Figure 5, lanes 1, 3, 5). The control included cDNA reaction mixtures with RNA but no reverse transcriptase (Figure 5, lanes 2, 4 and 6) and without RNA (Figure 5, lane 9). Lanes 7 and 8 (lane 8 is a negative control of lane 7 without the reverse transcriptase) show PCR products of 40 cycles amplification using β-actin primers from total RNA of human nasal mucosa. These results indicated the existence of TRPV1 mRNA in human nasal mucosa, especially in nasal epithelium and vascular endothelium.

DISCUSSION

In the present study, we demonstrated the localization and the expression of TRPV1 in human nasal mucosa at both protein and mRNA level by using immunohistochemistry and RT-PCR, respectively. TRPV1 positive cells in human nasal mucosa were found on epithelial cells, vascular endothelial cells, submucosal glands and nerves.

It was previously thought that TRPV1 existed only in neuronal cells, such as dorsal root ganglia [3], trigeminal ganglia [19], brain [20] and so on. Recently the presence of TRPV1 has been recognized in non-neuronal cells as well: cardiomyocytes [7], bronchial epithelial cells [8], urinary bladder epithelial cells [9], gastric epithelial cells [10,11], oral epithelium [12] and keratinocytes [13,14]. These observations lead to the assumption that TRPV1 distribution is more broad than previously thought, however, a physiological role for these receptors in non-neuronal cells has not been established.

The mechanisms of reduction of nasal symptoms by repetitive pretreatment of capsaicin are reported to be desensitization of the nasal mucosa [5]. It is furthermore suggested that neuropeptides play some important roles in nasal neurogenic inflammation, including allergic rhinitis. Depletion of neuropeptides can partly explain why capsaicin pretreatment to the nasal mucosa improves the nasal symptoms.

This study shows TRPV1 expression in nasal epithelium and vascular endothelial cells. Agopyan et al. [21] reported that the primary cultures of upper and lower human airway epithelial

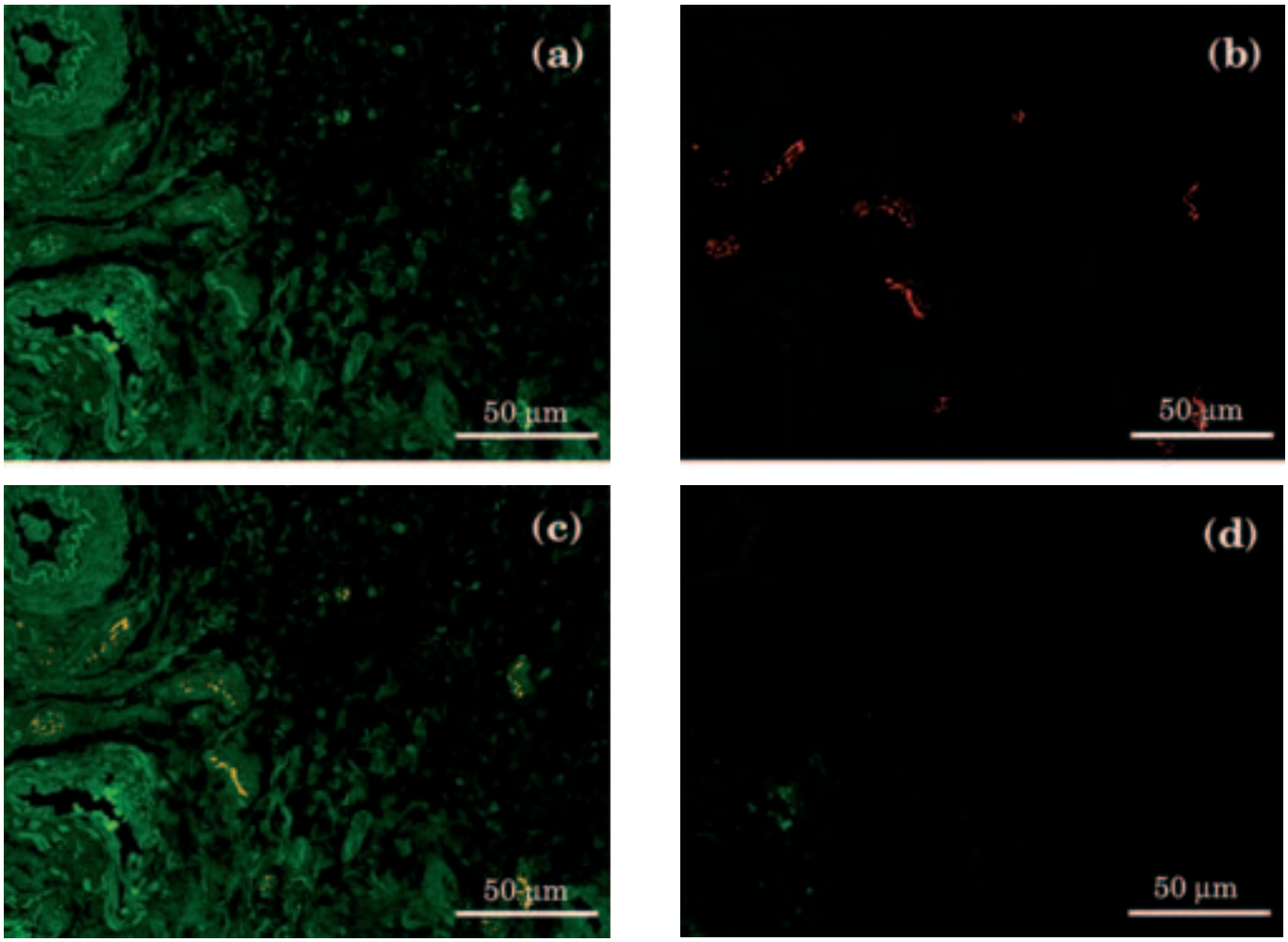

Figure 3. Immunofluorescence showing colocalization of expression of TRPV1 and neurofilaments. (a) Expression of TRPV1 (green) (b) Expression of neurofilaments (red) (c) Colocalization is demonstrated by the yellow of the superimposed images of the double staining. (d) Negative control.

cells (NHBE: normal human bronchial/tracheal epithelial cells and SAEC: normal human small airway epithelial cells) expressed the TRPV1 gene. Several studies showed that capsaicin, particulate matters and neuropeptides acted to promote the production of various cytokines (IL-6, IL-8 and TNF-α) by human respiratory epithelial cells [8, 22-24]. Furthermore, it is reported that anandamide, an endogenous TRPV1 ligand, induced apoptosis in a time- and dose-dependent manner in HUVECs (human umbilical vein endothelial cells). This endothelial cell death was inhibited by capsazepine, a TRPV1 antagonist, and was enhanced by capsaicin. These results suggest that capsaicin stimulates TRPV1 on the epithelial and the endothelial cells of the nasal mucosa and regulates the production of cytokines and apoptosis of the endothelial cells, which may be another function of TRPV1 on the upper airways.

Several reports showed that capsaicin had the gastroprotective action mediated by the activation of TRPV1. The mechanism of this gastric protection is thought to be associated with an increase in mucosal blood flow [25, 26], the secretion of mucus [27] and bicarbonate [28] through TRPV1-sensitive sensory neurons. However, this protective action is also observed in a cultured rat gastric epithelial cell line (RGM-1), devoid of neurons or blood vessels. Kato et al. [10] suggested that capsaicin might act as cellular protection in RGM-1 through an increase in mucus secretion mediated by Ca^{2+} on the following bases: (1) cultured gastric mucosal cells secrete mucus in response to Ca^{2+} or cAMP [29,30], (2) TRPV1 acts as a nonselective cation channel with high permeability especially for Ca^{2+}, (3) TRPV1 responds to agonists by increasing the intracellular Ca^{2+} concentration [3]. In our study, TRPV1 positive cells were found among vascular endothelial cells, submucosal glands and nerves, and moreover goblet cells and mucous cells of the submucosal glands expressed higher levels of TRPV1 immunoreactivity. Although the precise role of TRPV1 in goblet cells and submucosal glands is unclear, these findings lead us to assume that capsaicin may directly influence the secretory function through activation of TRPV1 on the human upper airways.

In conclusion, we have demonstrated the localization and the expression of TRPV1 in human nasal mucosa using immunohistochemistry and RT-PCR. The role of TRPV1 in the upper airway has not been fully clarified. However, precise knowl-

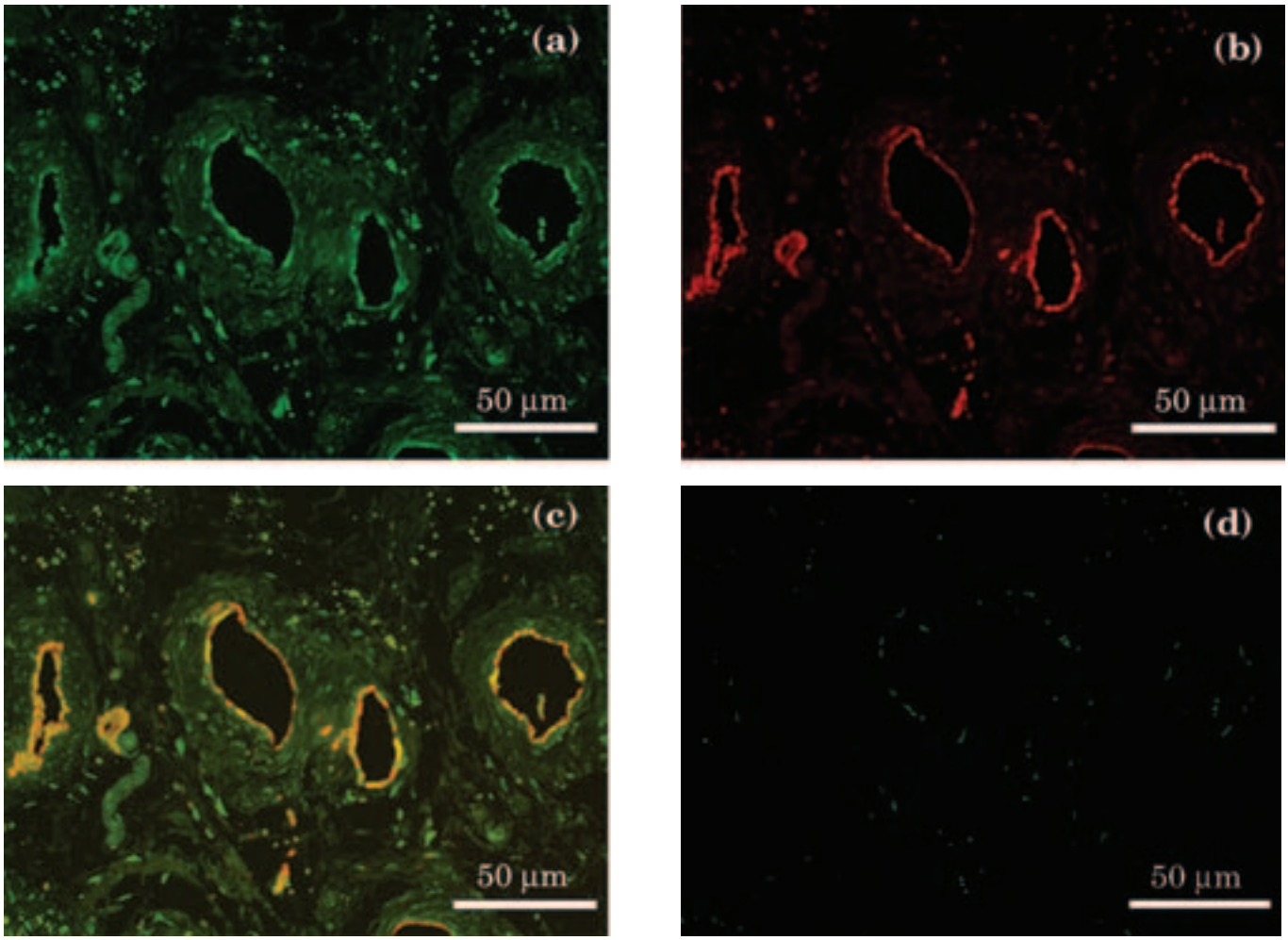

Figure 4. Immunofluorescence showing colocalization of expression of TRPV1 and CD31. (a) Expression of TRPV1 (green) (b) Expression of CD31 (red) (c) Colocalization is demonstrated by the yellow of the superimposed images of the double staining. (d) Negative control.

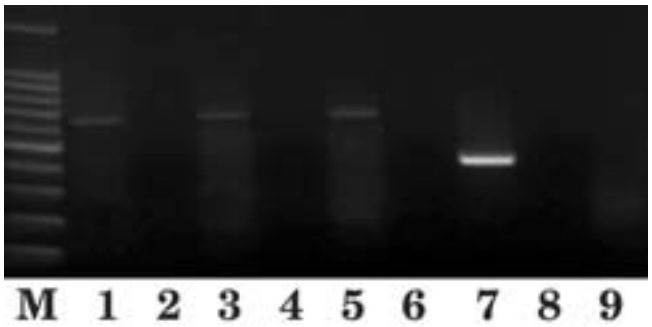

Figure 5. Detection of TRPV1 mRNA by RT-PCR for 40 cycles of amplification from human nasal mucosa and cultured cells. RT-PCR was performed using TRPV1 primers and ,-actin primers, demonstrating 676 bp and 403 bp fragments respectively. Lane M: 100 bp ladder. Lane 1: PCR products of 40 cycles amplification using TRPV1 primers from total RNA of human nasal mucosa. Lane 2: negative control of lane 1 (no reverse transcriptase). Lane 3: PCR products of 40 cycles amplification using TRPV1 primers from total RNA of epithelial cultured cells. Lane 4: negative control of lane 3 (no reverse transcriptase). Lane 5: PCR products of 40 cycles amplification using TRPV1 primers from total RNA of cultured vascular endothelial cells. Lane 6: negative control of lane 5 (no reverse transcriptase). Lane 7: PCR products of 40 cycles amplification using β-actin primers from total RNA of human nasal mucosa. Lane 8: negative control of lane 7 (no reverse transcriptase). Lane 9: negative control (without RNA sample, no reverse transcriptase).

edge of the identity and the distribution of TRPV1 should be of great assistance to understand upper airway diseases including allergic rhinitis. TRPV1-expressing cells in the upper airway may be further therapeutic targets for these diseases.

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