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ORIGINAL ARTICLE

Expression of galanin and galanin receptor mRNA in skin during the formation of granulation tissue

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Abstract Galanin is a neuropeptide widely distributed in the central and peripheral nervous systems. Although its role in non-neural cells is poorly understood, it is known that during inflammation, the dermis layer of the skin produces and releases galanin. The aim of this report is to study the expression of galanin in granulation tissue. After inducing inflammation by cotton thread implantation, galanin-like immunoreactivity (galanin-LI) in plasma reached a maximum on the third day. Galanin-LI was observed in fibroblast-like cells occurring close to collagen fibers in developing granulation tissue. Furthermore, galanin receptor subtypes 1 and 2 (GALR1 and GALR2)-expressing cells were observed around microvessels and were found to produce desmin. Galanin was injected along the cotton threads immediately after implantation, resulting in rapid formation of granulation tissue, and an increase in the contents of microvessels, indicating a stimulatory effect of galanin on the process of angiogenesis in granulation tissue. The results demonstrate that some galanin was released from fibroblast-like cells during the formation of granulation tissue, and that it stimulated angiogenesis.

Keywords Galanin · Fibroblast · Angiogenesis · Granulation · Pericyte

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Introduction

Galanin, a neuroendocrine peptide isolated from porcine intestine, is widely expressed in both the central and peripheral nervous systems [1–8]. Galanin has regulatory roles in several diverse processes, such as nociception, hormone and neurotransmitter release, and spinal reflexes, acting via the three galanin receptors that are known to exist: GALR1, GALR2, and GALR3 [9, 10]. Studies have shown that all three receptor types are involved in the various regulatory roles of galanin mentioned above.

In the peripheral nervous system, axotomy [11–15] and chronic nerve compression [15–17] induce galanin expression in dorsal root ganglion (DRG) neurons, and galanin in the spinal cord is linked to the modulation of pain [9, 18–20]. Recently, it has been suggested that galanin plays mitogenic and neurotrophic roles that may be of importance in recovery from neuronal injury [21].

Recently, it has become apparent that galanin is also expressed in non-neural cells. In human skin, galanin-like immunoreactivity (galanin-LI) was first reported in nerve terminals and fibers of the dermis [22, 23]. More recently, galanin-LI has been detected in keratinocytes [24], the follicular and interfollicular epidermis, and in the ductal cells of eccrine sweat glands [25]. In addition, galanin-binding sites have been observed around eccrine sweat glands [25] and in the basal zone of the epidermis [23]. Regarding the effects of galanin acting on GALRs in non-neuronal cells, galanin induces an up-regulation of inflammatory cytokine (IL-1α) and chemokine (IL-8) production in keratinocytes [26], and inhibits proliferation via an action on GALR1 and GALR3 in immature rat thymocytes [27], implying that galanin plays an important role in inflammatory processes. Such processes are complex biological events in which a large number of resident and

infiltrating cells are involved. These cells fulfill immunological roles, and regulate each other by releasing a variety of cytokines and growth factors. The aim of this study is to investigate the expression of galanin and galanin receptors in skin, and the effect of galanin on granulation tissue and angiogenesis.

Materials and methods

Animals

All experimental protocols were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka. Male Wister rats were purchased from Nippon SLC Ltd. (Shizuoka, Japan) and bred under conventional conditions in a temperature- and humidity-controlled environment with a 12-h light/dark cycle. Experimental rats were given with standard food and water ad libitum until the time of experiment.

Induction of granulation tissue by use of cotton threads

A piece of cotton thread was soaked overnight in ethyl acetate and dried at room temperature. Male rats (7-weeks-old) were anesthetized, and a thread (1 cm in length, weighing 7 mg) was implanted subcutaneously into the dorsum with a 13-G implantation needle (Natsume Co.), using a modified version of a previously established procedure [28]. To assess the effects of galanin on granulation tissue formation and angiogenesis, rat galanin (100 ng/100 μl saline) was subcutaneously injected into the site of the cotton thread implantation immediately after implantation, and then once every day thereafter.

Isolation of fibroblast-like cells from granulation tissue

The granulation tissue was cut into pieces ~1 mm in diameter. The pieces were cultured in 35-mm Petri dishes. Fibroblast-like cells were outgrowth from explants. These cells were cultured in 10 ml of RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS, Moretate Biotech, Bulimba, Australia) in the presence of 5% (v/v) CO_2 under 100% (v/v) humidity. When cultures achieved 80% confluency, cells were used in the experiment.

Determination of granulation tissue weight and assessment of angiogenesis in granulation tissue

Rats were anesthetized with diethyl ether and killed by dislocation of the cervical spine. The granulation tissue formed was dissected out of the dorsum, together with the cotton thread, and weighed. Angiogenesis was assessed by measuring the hemoglobin content of the granulation tissue [29]. The excised granulation tissue was washed in PBS and homogenized in 1 ml of 0.5 mM sodium hydroxide, and then placed on ice. The tissue homogenates were centrifuged at 10,000 rpm at 4°C for 30 min. The supernatants (100 μl) were diluted in 100 ml of 0.5 mM sodium hydroxide, to which 20 μl of 2% (w/v) potassium ferricyanide and 20 ml of 0.5% (w/v) sodium cyanide were added. After 30 min of incubation, the hemoglobin concentration was then determined spectrophotometrically by measuring the absorbance at 550 nm.

Radioimmunoassay

Galanin-LI was determined by radioimmunoassay (RIA) using rabbit anti-rat galanin (1–15) serum R0672 (final dilution 1:21,000), as described in a previous report [30, 31]. The doses of the standard antigen that displaced 10% of the total antibody-bound antigen ranged from 5 to 640 fmol/tube.

Plasma and granulation tissue preparation for galanin RIA

One ml blood samples were collected from the caudal vein at 1, 3, 5, 7, and 10 days after thread implantation. Samples were centrifuged at 10,000 rpm for 10 min, and the plasma was recovered and stored at −80°C. One hundred microlitre of plasma was measured for galanin specific RIA. Each granulation tissue sample was placed in 5 ml of 0.1 M acetic acid and boiled for 10 min. Samples were allowed to cool, and then homogenized and centrifuged at 10,000 rpm for 30 min. The supernatants were de-collagenated by ultrafiltration (MWCO 30,000, Vivaspin cartridge, Vivascience, Germany). The samples were lyophilized and stored at −80°C prior to RIA. The extracts were dissolved in 0.01 M phosphate buffer (pH 7.4), 0.14 M NaCl, 0.025 M EDTA, and 0.5% (w/v) bovine serum albumin (BSA) and measured for galanin-specific RIA.

RT-PCR analysis

Total RNA was extracted from granulation tissue, fibroblast-like cells and the hypothalamus using an SV Total RNA Isolation System (Promega Corporation, USA). The total RNA extracted from the hypothalamus was used as a positive control [32–35]. Genomic DNA was removed with DNase I. cDNA synthesized by Ready-To-Go You-Prime First-Strand Beads (GE Healthcare UK Ltd, England) according to the manufacturer's instructions. After various dilutions of template cDNA, amplification by PCR using pure Taq Ready-To-Go PCR Beads (GE Healthcare UK Ltd, England) did not

reach plateau, but could still be used for semiquantitative analysis. Gene-specific primers were designed, as described in previous research [36], to amplify the progalanin (5′-GACCTGCACTAACCAGCTACG-3′ and 5′-GGTCTCAGGACTGGAGATTC-3′), GALR1 (5′-AACCTCCAGGGGAACCCAGG-3′ and 5′-CATAGCGATCCACAGACATCG-3′), GALR2 (5′-CCTCGAAACGCGCTGGCC-3′ and 5′-CTGTGCAGTTGGGAAGTG-3′) and GALR3 (5′-CTGTGGCAGTGCCTGTGATC-3′ and 5′-TGCCGCACAGCCAGGTACCT-3′. PCR cycles were performed as follows: initial denaturation at 95°C for 5 min was followed by 30 to 45 cycles (depending on the primer) at 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. PCR products were electrophoresed on ethidium bromide-containing 1.2% agarose gels and visualized under UV illumination.

Histochemistry

Rats were killed by dislocation of the cervical spine the day after cotton thread implantation. The granulation tissue was dissected out and fixed with 4% (w/v) paraformaldehyde in PBS, which was then replaced with PBS containing 30% sucrose, and embedded in OCT compound (Sakura Seiki, Tokyo, Japan) at −80°C. 7 μm thick sections were cut in a cryostat (model 1720, Leitz, CA, USA) at −21°C. The sections were dried at room temperature, and then hematoxylin–eosin staining was performed.

Immunohistochemistry

After blocking with normal goat serum (Vector Laboratories, CA, USA, ×100), the sections were incubated with monoclonal antibody B4G9, raised against rat galanin (1–15) (established in our laboratory, 1 μg/ml, specificity shown in supplemental Fig. 1) or anti-desmin monoclonal antibody (Dako Co., Carpinteria, USA, × 50) overnight at 4°C in a humid atmosphere. The anti-galanin(1-15) monoclonal antibody B4G9 was preincubated with 5 μg/100 μl of galanin for 1 h at room temperature and used as an adsorption control. The sections were sequentially incubated with rhodamine-conjugated anti-mouse IgG goat antibody (Biosource International, Camarillo, CA, ×500) for 1 h at room temperature. The sections were mounted with 80% (v/v) glycerol in PBS and coverslipped. The samples examined by a laser scanning confocal microscope (LSM510, Carl Zeiss, Co. Ltd., Oberkochen, Germany).

In situ hybridization

The GALR1 and GALR2 templates were prepared by RT-PCR using rat hypothalamus tissue. GALR1 and GALR2 specific primers were used as described in above. The PCR products were purified with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd, England). The biotinylated DNA probes were made with a Detector Random Primer DNA Biotinylation Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) using random hexamer primers and biotin-N4-dCTP. The granulation tissue was fixed with 4% (w/v) paraformaldehyde in PBS, which was then replaced with PBS containing 30% sucrose, and embedded in OCT compound at −80°C. 7 μm thick sections were incubated sequentially with 0.1 M triethylamine in 0.25% (v/v) acetic acid, 4% (w/v) paraformaldehyde and 1 mg/ml proteinase K. The biotinylated DNA probe (1 μg/ml) diluted with hybridized buffer was then hybridized with the sections for 18 h at 42°C in a humid atmosphere. The biotinylated DNA probe was preincubated with GALR1 or GALR2 templates (10 μg/ml) for 30 min and used as a negative control. After blocking with BlockAce (DS Pharma Biomedical, Japan) for 30 min, FITC-avidin D (Vector Laboratories, CA, USA, ×250) was added to the hybridized sections for 1 h at room temperature. The sections were mounted with 80% (v/v) glycerol in PBS, coverslipped, and then observed with a laser-scanning confocal microscope.

Data analysis and statistics

Numbers of samples analyzed and experiments performed are indicated in the figure legends. Data are presented as means ± SEM from representative experiments. Tukey–Kramer tests were used to determine the significance ($P < 0.05$) of differences between data sets.

Results

Cotton thread-induced granulation tissue and galanin concentration in plasma

Subcutaneous implantation of cotton threads into the dorsum of rats induced gradual increases in the weight of the granulation tissue from days 1 to 3 after implantation (Fig. 1a). The concentration of galanin-LI in plasma rose to ∼130 fmol/ml 3 days after implantation, then gradually decreased to its basal level after 5 days (Fig. 1b). Galanin-LI was detected in granulation tissue extracts (318 ± 58 fmol/g granulation tissue). Galanin concentrations in the skin of control rats, in which threads had not been implanted, were very low compared with galanin concentrations measured in the skin of rats 3 days after cotton thread implantation (Fig. 1c).

Expression of galanin and galanin receptors in granulation tissue

Expression of galanin and galanin receptors was determined using RT-PCR analysis. In untreated dermis tissue, galanin

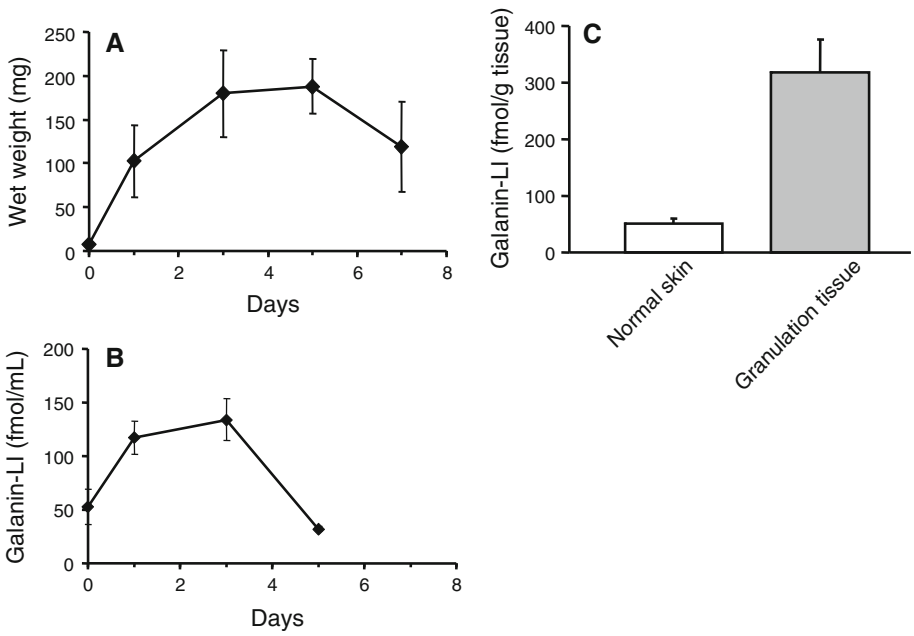

Fig. 1 Cotton thread-induced granulation tissue and angiogenesis. Subcutaneous implantation of a cotton thread into the dorsum of rats induced gradual increases in the weight (**a**), and levels of galanin-LI in plasma (**b**) and granulation tissue (**c**). The plasma galanin-LI levels increased to a maximum around days 1–3, and returned to normal levels by day 5. Galanin-LI was detected in granulation tissue. In the Figure above, each point and bar represents mean ± SE ($n = 3$–4)

mRNA was detected at very low levels. Three days after implantation of cotton threads, however, galanin, GALR1 and GALR2 mRNAs were expressed in granulation tissue, but GALR3 mRNA was not detected (Fig. 2a). To reveal the location of the galanin-LI and GALRs in granulation tissue, we used immunohistochemistry for galanin, and in situ hybridization for the GALRs. In granulation tissue, galanin-LI positive cells were detected (Fig. 2b). Galanin-LI positive cells were diminished using B4G9 pre-absorption with galanin (Fig. 2c). Cells exhibiting galanin-LI were located in granulation tissue close to collagen fibers, and resembled fibroblast-like cells. In addition, galanin mRNA was expression in fibroblast-like cells from granulation tissue (Fig. 2d). GALR1 or GALR2 mRNA-expressing cells were observed in granulation tissue using in situ hybridization. Consequently, granulation tissue was double-stained with an antibody against desmin, a marker of pericytes. GALR1 or GALR2 mRNA were expressed in granulation tissue, and GALR1 and GALR2-expressing cells located around microvessels (Fig. 2e, g). In contrast, using GALR1 or GALR2 probes preincubated with GALR1 or GALR2 templates, GALR1 or GALR2 positive cells were not detected (Fig. 2f, h). In addition, some cells expressing GALR1 or GALR2 mRNA were found to contain desmin (Fig. 2e, g). This indicated that galanin might stimulate angiogenesis via an action on pericytes during granulation tissue formation.

The effects of galanin injection on granulation tissue and angiogenesis

To clarify the role of galanin in granulation tissue formation and angiogenesis, rat galanin was subcutaneously injected into the site of cotton thread implantation immediately after implantation, and once every day thereafter. This resulted in a rapid development of granulation tissue, compared with that seen after the injection of saline as a control (Fig. 3a). A single injection of galanin was found to be enough to stimulate granulation tissue formation. The injection of rat galanin also increased hemoglobin levels 1–2 days after thread implantation (Fig. 3b). In addition, the increment of angiogenesis was investigated by histology. Microvessels were observed in granulation tissue (Fig. 3c). In the granulation tissue treated with galanin, there was an increase in the contents of microvessels (Fig. 3d). These findings suggest that the weight of cotton thread-induced granulation tissue, and the concurrent angiogenesis that it produced, are initially dependent on the action of galanin.

Discussion

Some neuropeptides, including substance P, neurokinin A, CGRP, VIP, neuropeptide Y, POMC peptide, ANP, somatostatin and galanin, have been detected in skin and skin-derived cells [23, 36–40], and can regulate inflammatory responses in the skin. It has been reported that some fibroblasts produce neuropeptides, such as adrenomedullin [41], substance P [42] and proopiomelanocortin-derived peptides (including α-MSH, ACTH and β-endorphin) [43]. Fibroblasts are located close to the extracellular matrix, and secrete matrix components such as collagen and laminin, as well as several cytokines, and play an important role in granulation tissue growth [44]. In this study, we

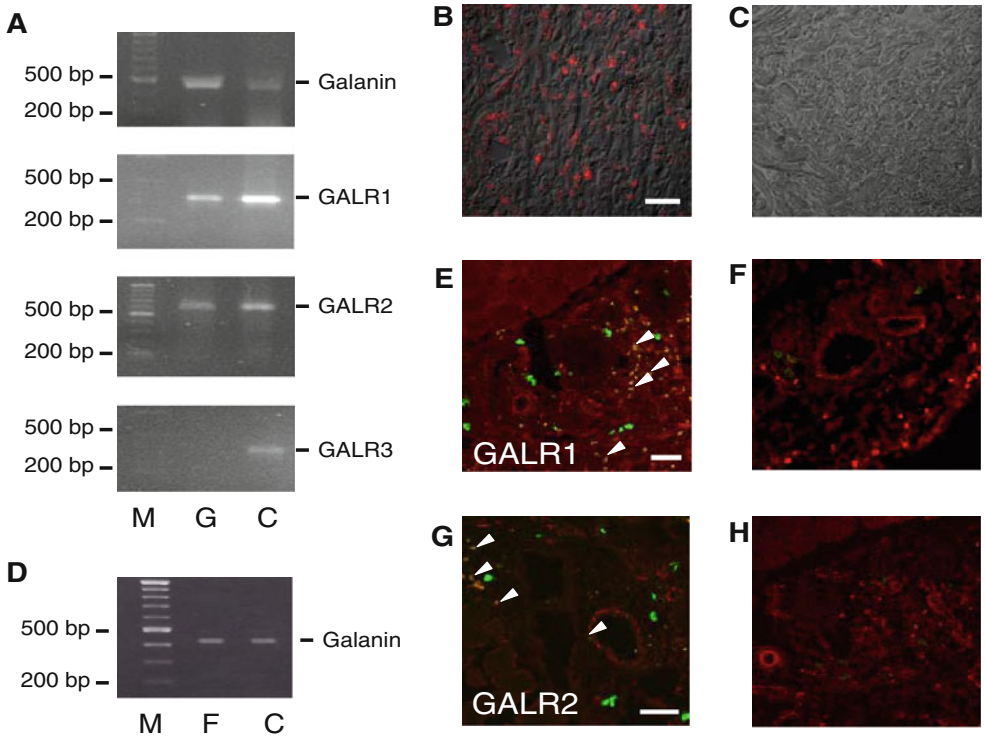

Fig. 2 Detection of galanin and GALRs mRNA in granulation tissue. Total RNA was extracted from the granulation tissue, reverse-transcribed, and amplified with specific primers against galanin (425 bp, 30 cycles), GALR1 (350 bp 40 cycles), GALR2 (672 bp, 40 cycles) and GALR3 (327 bp, 45 cycles) followed by 1.2% agarose gel electrophoresis (*M* marker, *G* granulation tissue, *C* positive control). **a** Immunostaining with anti-galanin (1–15) monoclonal antibody (B4G9) followed by rhodamine-anti-mouse IgG (*red*) in granulation tissue was shown. Tissue sections were examined with a confocal laser-scanning microscope (**b**). Absorption control in immunohistochemistry using rat galanin (**c**). *Scale bars* indicate 50 μm. RT-PCR analysis of galanin mRNA expression (425 bp, 40 cycles) in fibroblast-like cells from granulation tissue (**d**) (*M*, marker, *F* fibroblast-like cell, *C* positive control). In situ hybridization of GALR1 (**e**) or GALR2 (**g**) cDNA probes using FITC-avidin (*green*) and anti-desmin monoclonal antibody followed by rhodamine-anti-mouse IgG (*red*) in granulation tissue. The negative controls were performed with GALR1 (**f**) and GALR2 (**h**) cDNA probe preincubated with GALR1 or GALR2 templates. Some cells expressing GALR1 or GALR2 mRNAs were localized in desmin-positive cells (*yellow*, indicated by *arrowheads*). *Scale bars* indicate 100 μm

demonstrated that fibroblast-like cells produced and released galanin during granulation tissue formation. However, galanin was expressed at low levels in the normal skin. On the other hand, it has been reported that the expression of galanin-LI and galanin mRNA is induced by several inflammation such as carrageenan-induced inflammation in the epidermis [23] and dermis, and allergic contact dermatitis in nerve-fibers [45]. These results suggest that fibroblast-like cells were activated by various inflammatory mediators, leading to the expression of galanin, and that fibroblast-like cells were one of the source cells releasing galanin in the skin.

In addition, GALR expression in non-neuronal skin cells has been described in terms of the detection of galanin-binding sites in the epidermis and dermis of rat hind paw [25]. In normal skin, galanin-binding sites were observed in the basal layer of the epidermis, and inflammation was found to increase galanin-binding markedly [24, 25]. In recent years, it has become apparent that GALR2 are expressed in cultured human keratinocytes, and that application of galanin results in an increase in cytosolic [Ca^{2+}] [23, 46]. Furthermore, galanin induces an up-regulation of inflammatory cytokine and chemokine production, which can influence the expression and secretion of proform nerve growth factor (proNGF) and nerve growth factor (NGF) from keratinocytes [26]. This study found that GALR1 and GALR2 mRNAs were expressed in granulation tissue, including in pericytes, but only in pericytes that had been dissociated from endothelial cells. Furthermore, galanin-stimulation accelerated angiogenesis and granulation tissue development. Pericytes are contractile cells that surround the capillary endothelium and are critical for the development and maintenance of a functional vascular network. Some angiogenic factors such as VEGF, bFGF, PDGF, and TGF-β affect pericyte activity and proliferation [47]. We demonstrated that galanin may act as a novel angiogenic factor via GALR1 and GALR2 in pericytes. However, GALR1 mRNA and GALR2 mRNA

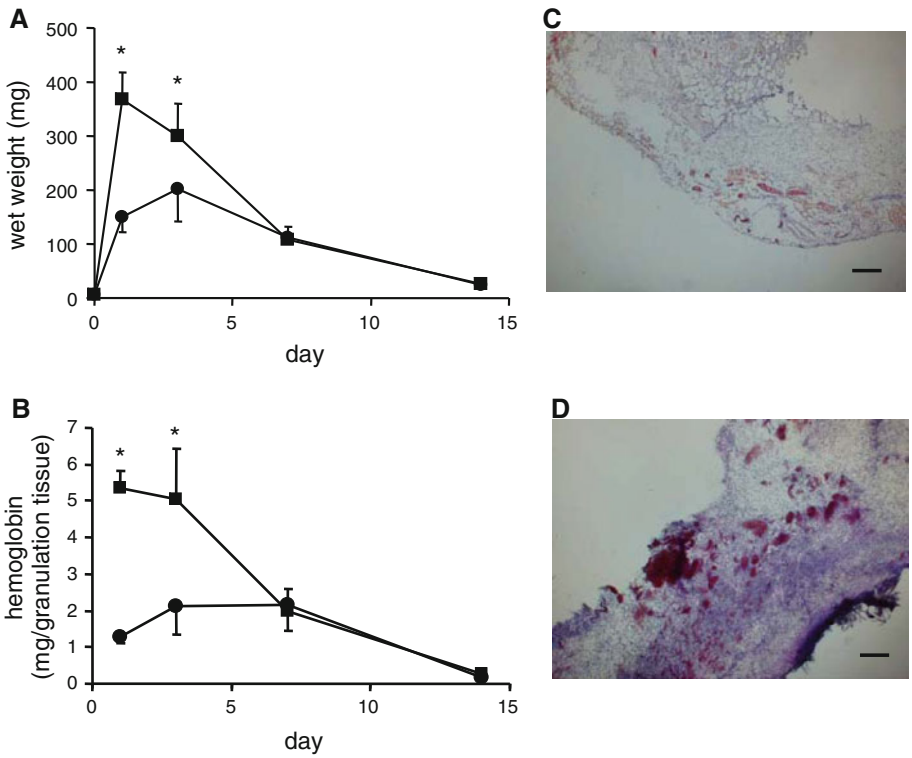

Fig. 3 The effects of galanin on granulation and angiogenesis. Galanin was injected along the cotton threads immediately after implantation. Granulation was assessed by weighing the excised tissue samples (**a**), and the extent of angiogenesis was determined by measuring the hemoglobin content (**b**). The cotton thread-induced granulation tissue weight and the extent of angiogenesis were stimulated by the injection of galanin in the early stages after thread implantation. *Squares and circles* represent granulation tissue treated with galanin and saline, respectively. Each point and bar represents mean ± SE ($n = 3$). *indicates significance at the $P < 0.05$. Histochemistry of granulation tissue treated without galanin (**c**) or with galanin (**d**) (100 ng/100 μl). Granulation tissues were dissected, sectioned and stained by hematoxylin and eosin staining. Scale bars indicate 200 μm

were only expressed in detached pericytes, while expression of any GALR types was undetectable in pericytes located around endothelial cells. Angiogenesis is triggered by the detachment of preexisting pericytes as a result of vascular destabilization, and galanin may play an ancillary role in the process of angiogenesis.

In conclusion, we have demonstrated that some galanin is released from fibroblast-like cells and plays an important physiological role in the early stages of granulation tissue formation and angiogenesis in pericytes.

Conflict of interest The authors declare no conflict of interest.

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