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Neuropeptides regulate expression of matrix molecule, growth factor and inflammatory mediator mRNA in explants of normal and healing medial collateral ligament

Paul Salo [a,*], Robert Bray [a], Ruth Seerattan [a], Carol Reno [a,b,d], Jason McDougall [c], David A. Hart [a,b,d]

[a] Department of Surgery, University of Calgary, Calgary, Alberta, Canada
[b] Department of Medicine, University of Calgary, Calgary, Alberta, Canada
[c] Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada
[d] Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada

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Abstract

Denervation degrades normal ligament properties and impairs ligament healing. This suggests that secreted neuromediators, such as neuropeptides, could be modulating cell metabolism in ligament and scar tissue. To test this hypothesis we investigated the effect of exogenous substance P (SP), neuropeptide Y (NPY) or calcitonin gene-related peptide (CGRP) on the mRNA levels for proteins associated with inflammation, angiogenesis, and matrix production in tissue-cultured specimens of normal and injured medial collateral ligament. SP and NPY induced increased mRNA levels for several inflammatory mediators in the 2-week post-injury specimens. All three neuropeptides induced decreases in mRNA levels for healing-associated growth factors and matrix molecules, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and collagen types I and III. The results indicate that neuropeptides strongly influence the metabolic activity of cells in healing ligament, particularly at early time points after injury.

Keywords: Ligament injury; mRNA; Substance P; Calcitonin gene-related peptide; Neuropeptide Y; Rabbit

1. Introduction

Significant joint injuries such as traumatic ligament disruption are among the most potent stimuli to joint inflammation [1]. It is now well accepted that the regional inflammatory response is markedly amplified by neurogenic factors [2–4]. After ligament or tendon injury, peptidergic neurons sprout and grow into the scar formed at the site, leading to speculation that neuronal derived factors could play an important role in connective tissue healing [5,6]. Neuropeptides stimulate endothelial and fibroblast proliferation *in vitro* [7,8] and angiogenesis *in vivo* [9,10].

Denervation can reduce the severity of inflammatory arthritis [3,11], but also significantly degrades normal ligament properties in the rat [12], and significantly impairs ligament healing, as measured by blood flow, angiogenesis and mechanical properties of the healing scar at 6 weeks after injury in a rabbit model [13]. Such observations suggest that secreted neuromediators, such as neuropeptides or other factors, could be modulating cell metabolism in the developing scar tissue to optimize the healing process.

Of the numerous peptides produced and secreted by neurons, substance P, CGRP and NPY are among the most widely distributed and best characterized, and all three are known to be present in normal ligament of rat and rabbit ([6] and unpublished data; [14,15]). Substance P is a vasodilator, increases capillary permeability, causes mast cell degranulation and is a potent leukocyte chemotactic agent [16,17]. CGRP is a potent

* Corresponding author. Tel.: +1 403 220 2159; fax: +1 403 270 0617.
E-mail address: salo@ucalgary.ca (P. Salo).

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vasodilator and a mitogen for endothelial cells (see Lundberg [18] for review). Neuropeptide Y, widely expressed by sympathetic neurons, is a vasoconstrictor, a potent stimulus to angiogenesis and a modulator of immune cell function [19,20].

Prior investigations have shown that neuropeptides can significantly affect gene expression in normal rabbit medial collateral ligament (MCL) tissue in explant culture [21], but the effect of exogenous neuropeptides on gene expression in injured ligaments has not been examined. Although denervation impairs ligament healing, the mechanism of this effect remains unknown. We hypothesized that neuropeptides could stimulate the production of matrix molecules and growth factors associated with repair.

2. Materials and methods

2.1. Animals

Adult New Zealand white rabbits ($n=18$) were used. 12 underwent bilateral MCL transection [22,23] and 6 were uninjured normals.

2.2. Explant culture

3 days or 2 weeks after injury, randomly selected rabbits were killed ($n=6$ injured and $n=3$ non-injured at each time point) and the MCLs removed, divided lengthwise into 2 segments (4 segments per animal) and placed into 1 mL of serum-free culture medium (DMEM, Gibco BRL) in 24-well culture plates. Two early time-points for assessment were chosen because it was thought that neuropeptides would likely have their greatest influence during the initial inflammatory phase of wound healing [24]. Each longitudinal segment contained a portion of the scar region as well as a segment of femoral and tibial ends of the ligament. After 24 h in culture, either SP, CGRP or NPY was added to the culture medium to a final concentration of 10^{-7} M, a concentration which has been found previously to influence fibroblast activity in culture [21,25]. For each rabbit, one segment served as a control, with no neuropeptide added to the culture medium. Normal ligaments from the uninjured rabbits were treated in the same manner as injured ligaments.

2.3. RNA isolation

After a further 24 h of culture at 37 °C in a humidified CO_2 incubator, the ligament fragments were retrieved and snap-frozen in liquid nitrogen until RNA extraction. Total RNA was extracted using the TRIspin method [26]. Briefly, the Trizol tubes were thawed and chloroform was added to each sample (300 μL/1 mL Trizol). Following extraction with chloroform, total RNA was further purified using the RNeasy spin extraction kit (Qiagen Inc., Mississauga, ON) with the addition of a DNAse step (RNAse-free DNAse, Qiagen Inc.) after the initial wash. RNA quantification was performed using the SYBR Green II dye (Molecular Probes Incorporated, Eugene, OR) on a Turner Model 450 Fluorometer (Barnstead/Thermolyne Corporation, Dubuque, IA) with excitation at 468 nm and emission at 525 nm using a standard curve of rRNA (Sigma Chemical). RNA was stored at −80 °C until used for RT–PCR analysis. Reverse transcription was carried out within 24 h of RNA extraction.

2.4. PCR primers

Rabbit-specific primer sets that have been developed and validated in previous studies were used throughout [21,23,25,27–29].

2.5. RT–PCR

Reverse transcription of 1 μg of total RNA was performed with the Omniscript RT–PCR kit (Qiagen) and aliquots of cDNA were amplified using rabbit-specific primer sets [23,25]. All samples in an experiment were subjected to reverse transcription at the same time to avoid potential variation, similarly all samples in an experiment were subjected to PCR at the same time. Collagen types I and III, biglycan and lumican mRNA levels were determined to assess matrix production, vascular endothelial growth factor (VEGF) and its cognate receptor VEGFR2, as well as plasminogen activator inhibitor-1 (PAI-1) expression were assessed as indices of angiogenesis activity. Transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (FGF2) mRNA levels were assayed as indicators of growth factor production. The mRNA levels for interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were also measured to indicate inflammatory activity.

PCR products were separated in 2% agarose gels at 60 V/cm in TAE buffer, the gels stained with ethidium bromide, destained with distilled water and imaged with the GelDoc XR system (BioRad). Automated densitometry was performed using Discovery Quantity 1 software (BioRad). The relative abundance of the various mRNAs in the healing ligament was determined after normalizing to values obtained for the housekeeping gene β-actin, which did not vary from normal values in the injured samples. RT–PCR analysis of a second aliquot of RNA was done for a subset of the genes of interest and it revealed the same pattern of results. All no RT controls were negative for genomic contamination of RNA preparations.

2.6. Statistical analysis

Results were analyzed by analysis of variance and paired *t*-tests using Microsoft Excel software.

3. Results

3.1. Effect of neuropeptides on mRNA levels of growth factors

As neuropeptides may promote ligament healing by stimulating increases in production of growth factors known to be expressed in healing ligament and connective tissues, mRNA levels for TGF-β1, bFGF and VEGF were assessed.

TGF-β1 mRNA levels were found to be significantly depressed in 2 weeks post-injury ligament specimens cultured with 10^{-7} CGRP and 10^{-7} NPY compared to untreated specimens (Tables 1–3). SP had no detectable effect on TGF-β mRNA levels in injured ligament. TGF-β1 mRNA levels in normal uninjured ligament and in 3-day post-injury ligament explants were not significantly affected by any of the neuropeptides employed.

The mRNA levels for bFGF were responsive to neuropeptide exposure, with significant depression detected at 3 days post-injury with NPY, and at 2 weeks post-injury with SP and CGRP.

The mRNA levels for VEGF were significantly depressed by CGRP and NPY only at 2 weeks post-injury.

3.2. Effect of neuropeptides on mRNA levels for angiogenesis-associated proteins

Plasminogen activator inhibitor-1 (PAI-1) mRNA levels were significantly depressed by CGRP and NPY in the 2-week post-injury specimens. Similarly, VEGF receptor mRNA levels were depressed by CGRP and NPY in the 2-week post-injury specimens.

Levels of angiogenesis inhibitor TSP-1 mRNA were not significantly changed by any of the neuropeptides tested in normal or injured ligament specimens (Tables 1–3).

3.3. Effect of SP, CGRP and NPY on mRNA levels for matrix molecules

Collagen type I mRNA levels were significantly depressed in normal ligament specimens exposed to SP, by CGRP in 3-day post-injury specimens and by CGRP and NPY in 2-week post-injury specimens.

Collagen type III mRNA levels were significantly depressed by all three neuropeptides in the 2-week post-injury specimens.

Biglycan mRNA levels were significantly lowered by CGRP in both the 3-day and 2-week post-injury specimens, and by NPY in the 2-week post-injury specimens.

Table 1
Effect of neuropeptides on normal ligament specimens in explant culture

Gene	SP		CGRP		NPY	
	% Control	p [a]	% Control	p [a]	% Control	p [a]
TGF-β	99±9	0.94	107±5	0.48	86±5	0.22
bFGF	**77±20**	**0.03**	**64±14**	**0.03**	79±40	0.55
VEGF	59±16	0.08	115±21	0.14	65±12	0.23
PAI-1	67±24	0.35	74±13	0.45	67±14	0.39
VEGFR	**41±10**	**0.03**	111±8	0.54	48±14	0.07
TSP-1	94±47	0.92	45±42	0.22	61±31	0.35
Col I	52±15	0.04	119±22	0.31	75±33	0.25
Col III	52±15	0.09	137±16	0.24	101±19	0.89
Biglycan	91±13	0.16	126±19	0.29	102±7	0.87
Lumican	60±13	0.06	84±13	0.36	**40±25**	**0.01**
TNF-α	74±5	0.35	76±14	0.38	55±11	0.15
IL-1	89±5	0.66	75±23	0.43	70±10	0.30
iNOS	71±14	0.37	73±8	0.28	51±13	0.15
COX-2	72±7	0.23	66±10	0.12	65±9	0.20

Values for untreated, uninjured explants (controls) were set at 100%. Statistically significant results are shown in bold.
[a] Compared to no peptide, p values determined by two-tailed paired t-test.

Table 2
Effect of neuropeptides on 3-day post-injury ligament specimens in explant culture

Gene	No peptide	SP		CGRP		NPY	
	% Control	% Control	p [a]	% Control	p [a]	% Control	p [a]
TGF-β	131±18	127±9	0.90	113±10	0.70	104±12	0.48
bFGF	135±25	52±30	0.08	77±24	0.11	**53±34**	**0.02**
VEGF	204±25	153±16	0.27	243±13	0.30	159±28	0.58
PAI-1	115±34	167±29	0.12	170±25	0.11	115±27	0.99
VEGFR	94±16	62±22	0.21	85±8	0.61	45±23	0.06
TSP-1	102±44	97±39	0.92	107±23	0.90	149±38	0.23
Col I	284±12	**205±23**	**0.05**	**387±16**	**0.03**	250±24	0.54
Col III	330±13	**188±20**	**0.02**	380±16	0.14	231±23	0.27
Biglycan	234±9	286±13	0.27	**330±13**	**0.02**	294±20	0.26
Lumican	115±11	88±15	0.07	119±13	0.70	100±13	0.48
TNF-α	207±22	247±26	0.18	321±51	0.51	142±28	0.29
IL-1	127±27	121±18	0.82	119±52	0.91	80±24	0.22
iNOS	92±34	100±29	0.83	175±54	0.42	76±30	0.55
COX-2	72±7	122±19	0.16	179±47	0.54	118±17	0.90

Values for untreated, uninjured explants (controls) were set at 100%. Statistically significant results are shown in bold.
[a] Compared to no peptide.

Lumican mRNA levels were significantly lowered by NPY in uninjured specimens but were not affected by any neuropeptide in any of the injured specimens (Tables 1–3).

3.4. Effect of neuropeptides on mRNA levels for inflammatory mediators

IL-1 mRNA levels were significantly increased by NPY and SP in the 2-week post-injury specimens.

Similarly, NPY increased mRNA levels for both TNF-α and COX-2 in 2-week post-injury specimens.

None of the neuropeptides tested had a significant effect on iNOS mRNA levels in these specimens (Tables 1–3).

Table 3
Effect of neuropeptides on 2-week post-injury ligament specimens in explant culture

Gene	No peptide	SP		CGRP		NPY	
	% Control	% Control	p [a]	% Control	p [a]	% Control	p [a]
TGF-β	154±3	151±9	0.75	**50±11**	**0.000006**	**83±10**	**0.0006**
bFGF	207±18	**112±19**	**0.05**	**104±25**	**0.04**	122±35	0.06
VEGF	426±12	278±21	0.11	**180±17**	**0.004**	**133±10**	**0.002**
PAI-1	366±9	407±6	0.20	**201±8**	**0.002**	**242±22**	**0.008**
VEGFR	127±11	**63±29**	**0.04**	**70±20**	**0.006**	**43±4**	**0.0006**
TSP-1	167±24	111±28	0.40	127±16	0.1405	129±41	0.28
Col I	795±8	654±11	0.15	**315±16**	**0.002**	**262±13**	**0.0003**
Col III	698±10	**477±13**	**0.02**	**297±13**	**0.0003**	**157±13**	**0.0002**
Biglycan	418±13	402±10	0.67	**204±11**	**0.006**	**218±11**	**0.007**
Lumican	223±7	205±12	0.42	210±15	0.57	184±11	0.07
TNF-α	260±12	395±23	0.14	312±11	0.19	**475±16**	**0.009**
IL-1	147±12	205±12	0.10	**216±9**	**0.006**	**252±20**	**0.04**
iNOS	127±20	162±15	0.23	106±18	0.30	142±20	0.56
COX-2	140±11	257±24	0.07	154±10	0.47	**222±18**	**0.05**

Values for untreated, uninjured explants (controls) were set at 100%. Statistically significant results are shown in bold.
[a] Compared to no peptide.

4. Discussion

The studies presented in this report showed that the neuropeptides SP and NPY can induce increased mRNA levels for inflammatory mediators in specimens of injured ligament placed in culture at 2 weeks after injury. The importance of initial inflammation to successful wound healing is well documented, so these results are consistent with the idea that regulation of inflammatory mediator production is one of the major mechanisms by which neuropeptides influence the early phases of healing [30,31]. It is not clear why mRNA levels for iNOS were not affected by neuropeptide in this study as has been reported in other studies [32,33]. This may be a species-specific or tissue-specific observation.

In contrast, all three neuropeptides tested induced significantly lower mRNA levels for several molecules associated with healing in MCL scar, including growth factors, matrix molecules (excluding lumican) and some angiogenesis-associated proteins. Prior experiments in our laboratory revealed that denervation, an intervention which would be expected to deplete neuropeptides in the injury site, leads to increased mRNA levels for MMP-13, matrix components collagen types I and III, TGF-β and angiogenesis inhibitors TIMP-3, and TSP-1 in normal and healing ligament [34]. The present study is consistent with this and supports the idea that the effects of denervation are largely the result of the loss of neuropeptide stimulation. However, the results of both of these studies seem counter-intuitive in the context of our previously obtained *in vivo* data, which showed that denervation impaired ligament healing in the rabbit model [13].

There are a number of possible explanations that could account for this apparent paradox. The three neuropeptides tested stimulate increased blood flow and/or angiogenesis [9,20,35–37]. Given that angiogenesis is widely accepted to be the key determinant of the outcome of wound healing, neuropeptide-induced increases in blood flow and accelerated angiogenesis might well have a greater influence on the outcome of healing than the observed changes in mRNA levels for matrix molecules and growth factors [38–43].

Neuropeptides are also known to increase cellular proliferation [7,44–46]. Cells stimulated to proliferate would potentially downregulate or stop producing matrix molecules or growth factors until their proliferative phase was completed. Increased cellularity would likely subsequently lead to the formation of a larger, stronger scar *in vivo*, and could account for the superior healing of innervated ligaments.

Not all potentially important neuropeptides known to be present in articular tissues were tested. Vasoactive intestinal polypeptide (VIP), somatostatin (SOM) and met-enkephalin, known to be present in articular tissues [14,15,47,48], may also be important modulators of cellular metabolism in healing ligament. Future studies will address the influence of these mediators on healing and scar cell behavior, as the neuropeptide milieu in the healing ligament is likely very complex.

Most of the significant effects on mRNA levels observed in the present experiments were seen in the 2-week post-injury specimens. This corresponds to the late inflammatory and early proliferative phase of healing in the rabbit MCL. The way cells in the scar respond to neuropeptides appears to be time dependent, with different effects seen at different times after injury [49]. Testing specimens retrieved at longer intervals after ligament injury may reveal additional differences in response to neuropeptide stimulation.

In vivo, neuropeptides are produced in a temporally and spatially regulated manner by nerve fibers in close proximity to existing or newly forming vessels [15,50]. During development, angiogenesis in the skin is highly regulated by neuronal factors (see Mukouyama et al. [51] for review). It is not known if a similar relationship is recapitulated in healing ligament or other wounds. However, a highly localized downregulation of matrix production would facilitate angiogenesis, which depends on MMP-mediated matrix digestion to create a channel for proliferating endothelial cells to migrate into. Since all the neuropeptides tested in the present study are associated with increased blood flow and angiogenesis, the results support the conclusion that blood vessels and endothelium are likely the primary targets of these neuropeptides in early ligament healing [9,20,36,37,43].

There are a number of potential limitations to the study presented. An explant system was used and the specimens were all cultured for 24 hr prior to neuropeptide stimulation. We chose to do this to eliminate the effect of endogenous neuropeptides that might be present in the tissues at the time of tissue removal. However, the loss of normal *in vivo* mechanical stimulation could alter tissue responses to neuropeptide stimulation. Others have reported that loss of mechanical loading alters responsiveness of musculoskeletal tissues to hormones [52] and growth factors ([53]; see Ehrlich and Lanyon [54] for review) and impairs healing [55,56]. Therefore the unloaded mechanical environment of tissue culture may also influence responsiveness to neuropeptides, although the latency of this effect is not known. This possibility should be the focus of future studies.

Similarly, neuropeptides have potent effects on macrophages, monocytes and other circulating leukocytes found in substantial numbers in early stages of wound healing [57,58]. If neuropeptide effects on healing were mediated in part by effects on these cells, the loss of tissue perfusion *in vitro* would essentially eliminate these effects. Furthermore, only one concentration of each neuropeptide was tested. Although this dosage was chosen to induce a maximal response profile, the effects of lower concentrations might potentially be different on either normal or injured specimens. Despite the above limitations, the data clearly show that neuropeptides can significantly influence the metabolic activity of the cellular components of ligament scar tissue, particularly in the early stages of healing. Further *in vivo* experiments are planned to elucidate the specific effects of individual neuropeptides on ligament healing.

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