Analysis of Neuropeptides in Stretched Skin

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Background: Mechanical forces modulate wound healing and scar formation through mechanotransduction. In response to mechanical stimulation, neuropeptides are released from peripheral terminals of primary afferent sensory neurons, influencing skin and immune cell functions and increasing vascular permeability, causing neurogenic inflammation.
Methods: A computer-controlled device was used to stretch murine skin. C57Bl6 mice ($n = 26$) were assigned to a cyclical square-wave tensile stimulation for 4 hours or continuous stimulation for 4 hours. Stretched skin was analyzed for expression of the neuropeptides, substance P and calcitonin gene-related peptide, their receptors (NK1R and calcitonin gene-related peptide receptor component protein), and growth factors (nerve growth factor, transforming growth factor β1, vascular endothelial growth factor, and epidermal growth factor) using immunohistochemistry and real-time reverse-transcriptase polymerase chain reaction.
Results: Cyclical stimulation resulted in a significant increase in expression of neuropeptides and growth factors, whereas the corresponding peptide receptors were down-regulated. Transcription of neuropeptide mRNA was elevated in stretched skin, which proves that neuropeptides are released from not only peripheral terminals of nerve fibers but also resident skin cells.
Conclusions: The authors' results suggest that skin stretching may alter cell physiology by stimulating neuropeptide expression, and that cyclical mechanical force may be more effectively stimulating mechanosensitive nociceptors or mechanoreceptors (mechanosensors) on cells. (*Plast. Reconstr. Surg.* 124: 102, 2009.)

Mechanical forces modulate cutaneous wound healing,[1,2] scar formation, and remodeling,[3,4] yet the mediators of this response are not fully understood. Our previous studies demonstrate that waveform-specific skin stretching stimulates cellular proliferation and angiogenesis in skin.[2,5] We hypothesize that regulation and control of neuropeptides in skin may be an important mechanism in wound healing and scar formation. A better understanding of this mechanism may lead to the development of novel therapies for intractable cutaneous wounds and abnormal scarring, including keloids and hypertrophic scars.[4] This study investigates expression of neuropeptides and growth factors after waveform-specific skin stretching.

Neuropeptides are released from peripheral terminals of primary afferent sensory neurons[6–8] and various types of cells, including fibroblasts,[9] mast cells,[10] and keratinocytes.[11,12] It has been recently demonstrated that these cells can convert a mechanical stimulus into an electrical signal through mechanoreceptors (mechanosensors) such as mechanosensitive ion channels, and regulate cellular proliferation and angiogenesis.[13] Neuropeptides are expressed in skin and directly modulate the functions of keratinocytes, Langerhans cells, mast cells, microvascular endothelial

cells, fibroblasts, and infiltrating immune cells.[14,15] Among these neuropeptides, substance P and calcitonin gene-related peptide modify skin and immune cell functions, such as cell proliferation, cytokine production, antigen presentation, sensory neurotransmission, mast cell degradation, vasodilation, and vascular permeability.[16,17] These proinflammatory responses collectively cause neurogenic inflammation. Synthesis of these neuropeptides is regulated by nerve growth factor (NGF).[6,16] In this study, we apply continuous and cyclical stretch on murine skin to analyze the differential expression of substance P and calcitonin gene-related peptide, their receptors, and important growth factors such as transforming growth factor $\beta 1$ (TGFβ1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and NGF.

MATERIALS AND METHODS

Skin Stretching Device

To stretch murine skin, we used a computer-controlled device consisting of a stepper motor coupled with a planetary gear system and rack-and-pinion drive that moves a lightweight distractor (Fig. 1). The tensile force is transmitted to the skin through a bar-shaped load cell mounted on a movable arm that converts its mechanical deformation into an electric signal. This output signal was used as a part of a feedback mechanism for the device. Two removable feet were added under the arms of the device and designed to conform to the shape of the mouse dorsum.

The software used to control the device was created with the LabVIEW 7.1 program (National Instruments Corp., Austin, Texas). It offers the possibility of applying continuous or cyclical ten-
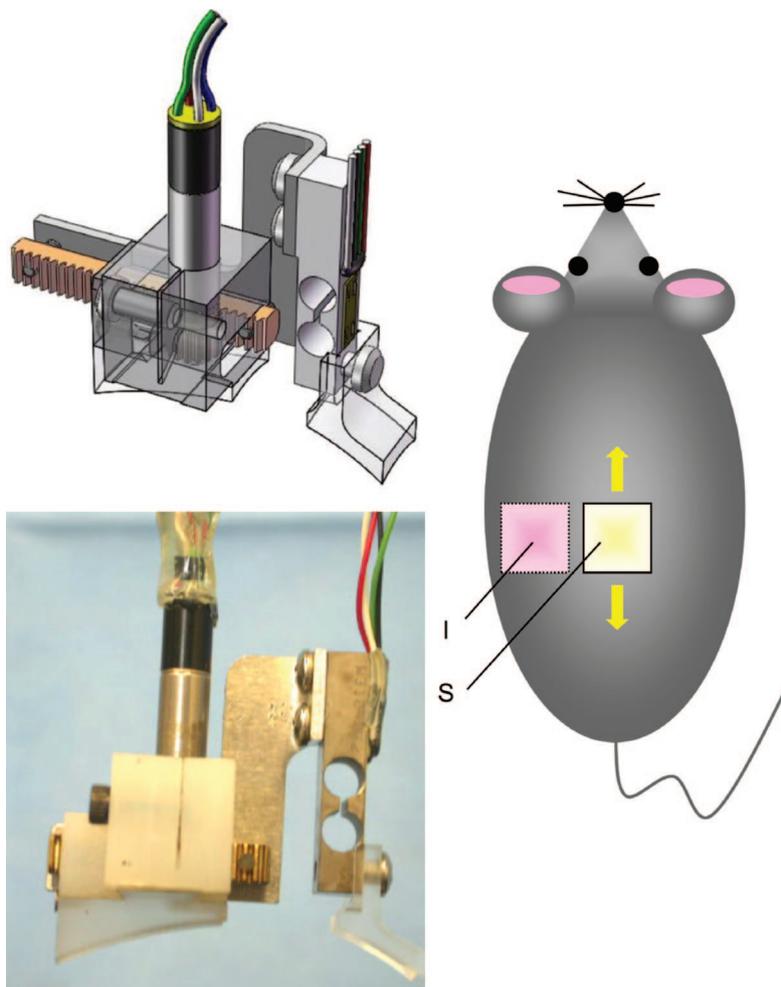
Fig. 1. A computer-controlled skin stretching device was used for this study. Two 1-cm² areas were designated: one was used for skin stretching and the other was used for internal tissue control. *I*, internal tissue control; *S*, stretched skin.

sile stimulation with adjustable variables such as length of cycles, intensity of applied forces, and number of cycles. The software records feedback signals and records them into Excel (Microsoft Office Excel; Microsoft Corp., Redmond, Wash.), permitting a log of the applied force.

Mouse Dorsal Skin Stretch Model

Twenty-six adult male C57Bl6 mice (Jackson Laboratory, Bar Harbor, Me.) were cared for under an approved experimental protocol in an Association for Assessment and Accreditation of Laboratory Animal Care–certified facility. The entire dorsum of the animals was shaved 24 to 72 hours before the beginning of each experiment; the shaving was superficial, leaving hair of a length of approximately 1 mm. A 1 × 5-cm transverse strip was depilated with Nair (Church & Dwight Co., Princeton, N.J.).

Ten minutes before application of the device, mice were anesthetized with intraperitoneal injection of 60 mg/kg pentobarbital (Nembutal Sodium Solution; Abbott Laboratories, Abbott Park, Ill.). Before application, we calibrated the device with a standard 50-g weight, and the two feet were positioned 1 cm apart. A layer of paper tape was attached underneath the feet of the device, and the two feet of the distractor were glued to the skin of the dorsum using Dermabond (Ethicon, Inc., Somerville, N.J.).

To verify the reliability of the applied force to the dorsal skin, the sensor log was analyzed after each application. The software registered the force amplitude at intervals of 1.5 seconds. Stimulations that did not follow the desired force pattern were omitted from the study. Digital photographs were taken before and after application of the devices. Photographs were analyzed carefully for macroscopic evidence of tissue damage.

Skin Stretching

Mice ($n = 26$) were assigned to a cyclical square-wave tensile stimulation for 4 hours ($n = 13$; 50 gf for 2 minutes and 0 gf for 1 minute) or continuous stimulation of a continuous 50 gf ($n = 13$) for 4 hours (Fig. 2). During stimulation, mice were able to move freely in their cages with unlimited access to water and food. At the end of each stimulation, the device was detached carefully from the mouse. All animals were caged separately.

Mice from each group were killed on day 2 ($n = 8$) and day 10 ($n = 5$) after stimulation. A $2 × 1$-cm skin sample was harvested from the stretch site of each mouse along with a matched sample from an area lateral to stretched skin, to be used as an unstretched internal tissue control. Each full-thickness sample was composed of skin and underlying subcutaneous tissue. The samples were cross-sectioned along the longitudinal stretch axis. In day-2 samples, five samples in each group were used for immunohistochemistry, and three samples were used for real-time reverse-transcriptase polymerase chain reaction analysis. Day-10 samples were only used for immunohistochemistry. For immunohistochemistry, samples were fixed in neutral buffered formalin 10% overnight, placed in alcohol 70% for 24 hours, and
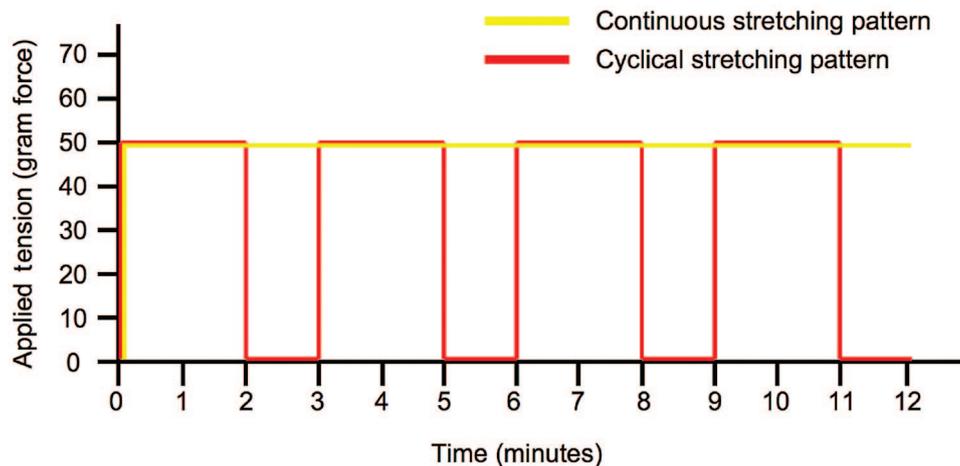
Fig. 2. The skin stretching pattern. In this study, mice ($n = 26$) were assigned to two different groups. The first group ($n = 13$) was subjected to a cyclical square-wave tensile stimulation for 4 hours. The pattern of this cyclical stimulation was 2 minutes on (50 gf) and 1 minute off (0 gf). The second group ($n = 13$) underwent continuous stimulation of 50-gf amplitude for 4 hours.

paraffin-embedded. For real-time reverse-transcriptase polymerase chain reaction analysis, freshly collected samples were washed with phosphate-buffered saline and frozen in Tissue-Tek optimal cutting temperature compound (Electron Microscopy Sciences, Hatfield, Pa.).

Immunohistochemistry

Analysis of accumulation of substance P, calcitonin gene-related peptide and expression of TGFβ1 and NGF in the skin was performed using immunohistochemistry. Buffered formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated by passage through xylene and graded ethanol solutions. After deparaffinization, slides were treated with 1% hydrogen peroxide in phosphate-buffered saline for 15 minutes, followed by pressure-cooker antigen retrieval at 125°C for 30 seconds and 90°C for 10 seconds in DAKO Target Retrieval Solution (DAKO, Carpinteria, Calif.). After 30 minutes of incubation with 10% goat serum to block nonspecific protein binding, first antibodies including rabbit anti–substance P (Chemicon International, Temecula, Calif.), rabbit anti-calcitonin gene-related peptide (American Research Products, Belmont, Mass.), rabbit anti-TGFβ1 (BioVision, Mountain View, Calif.), and NGF (Chemicon) were applied to sections at 1:1000, 1:100, 1:1000, and 1:100 dilution, respectively, overnight at 4°C. Then, peroxidase-labeled goat anti-rabbit immunoglobulin G (Vector Laboratories; Burlingame, Calif.) was applied as a second antibody for 1 hour at room temperature. Specimens were colorized by Nova Red (DAKO). Slides were counterstained with hematoxylin (Poly Scientific, Bay Shore, N.Y.).

Quantitative Analysis of Immunohistochemistry

Digital images were obtained in sequential fields at 40× magnification (Fig. 3). Quantification was performed by blinded observers. Hair follicles and vessels were omitted from all quantifications. Hypothetically, for unstretched skin compared with internal control, the baseline ratio is assumed to be 1.00. Values are expressed as means ± SD in text and figures, and the one-tailed t test was used to compare groups. Results were considered significant for values of $p < 0.05$.

Real-Rime Reverse-Transcriptase Polymerase Chain Reaction

Freshly collected samples were washed with phosphate-buffered saline and put into a small plastic tray with a small amount of Tissue-Tek optimal cutting temperature compound. Then, samples were frozen in liquid nitrogen and 20 μm of 10 sections was cut with a cryostat at $-20°C$. Then, total RNA was extracted using the RNeasy mini kit (Qiagen, Chatsworth, Calif.) following the manufacturer's instructions. Briefly, samples were finely homogenized with QIA shredder with buffer RLT including β-mercaptoethanol. After adding 70% ethanol and mixing well, samples were centrifuged using RNeasy spin column. Then, Buffer RW1 and RPE (Quiagen) were added sequentially into the column and centrifuged. Total RNA was extracted with RNase free water. Total RNA was quantitated using the NanoDrop (NanoDrop Technologies, Wilmington, Del.) method.

Complementary DNA was synthesized using a SuperScript III First-Strand Synthesis System for reverse-transcriptase polymerase chain reaction (Invitrogen Life Technologies, Carlsbad, Calif.).
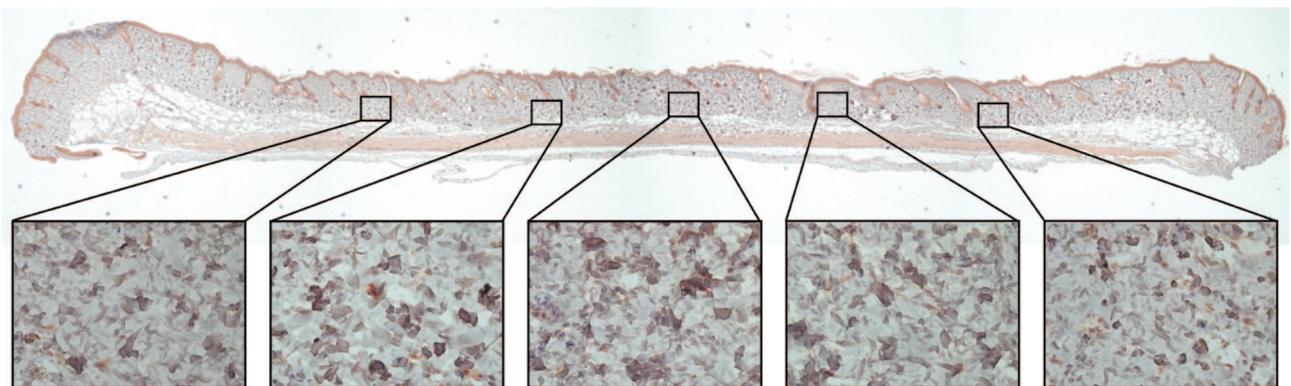
Fig. 3. Quantitative analysis of immunohistochemistry. Digital images were obtained from the middle of all stained skin sections. Positive stained cells were quantified using five sequential fields at 40× magnification. Hair follicles and vessels were omitted from all quantifications.

Total RNA (<1 μg) was mixed with random hexamers (50 ng/μl) and deoxyribonucleotide triphosphate (10 mM), then incubated at 65°C for 5 minutes. Tubes were cooled on ice, then reverse-transcriptase buffer (10×), magnesium chloride (25 mM), dithiothreitol (0.1 M), RNaseOUT (40 U/μl), and SuperScript III RT (200 U/μl) were added, giving a final volume of 21 μl. Samples were then incubated at 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 5 minutes, and cooled on ice. Then, *Escherichia coli* RNase H (2 U/μl) was add and incubated at 37°C for 20 minutes.

Real-time reverse-transcriptase polymerase chain reaction was performed in an ABI Prism7300 system (Applied Biosystems, Foster City, Calif.) using The RT^2 SYBR Green/ROX qPCR master mix (SA Biosciences, Frederick, Md.) with primers designed for this study (Table 1). Amplifications of the cDNA samples were performed in triplicate in 96-well plates in a final volume of 20 μl at 40 polymerase chain reaction cycles consisting of a denaturation step at 95°C for 15 seconds and an anneal/extension step at 60°C for 1 minute. Fluorescence measurements were used to generate a dissociation curve using the system software program v1.4 (Applied Biosystems) with glyceraldehyde-3-phosphate dehydrogenase as the endogenous control for normalization. Parameters used were as follows: automatic Ct calculation, automatic outlier removal, and relative quantity minimum/maximum confidence of 95.0 percent. When the baseline relative quantity minimum or maximum values did not overlap with the sample relative quantity values, the change in expression was considered significant.

RESULTS

Skin Stretching Model

For all stimulation groups, the device delivered the specified force patterns with minimal variation. Examples of feedback during cyclical and continuous stimulation patterns are shown in Figure 2. Macroscopic analysis of the dorsal skin revealed that the tensile force caused no damage to the stretched area of skin.

Immunohistochemistry

In the epidermis and dermis, both cyclical and continuous stimulation resulted in a significant increase in expression of substance P, calcitonin gene-related peptide, and growth factors including TGFβ1 and NGF at 2 days, returning to baseline at 10 days (Figs. 4 through 7). In addition,

Table 1. Primers for Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Gene	GenBank ID	Sense	Antisense
Tachykinin 1 (Tac 1)	D17584	5'-ACCTGCTCCACTCCTGCACCGCGGCCAAG-3'	5'-GAACTGCTGAGGCTTGGGTCTTCGGCGGAT-3'
Neurokinin receptor 1 (NK1R)	NM_009313	5'-GCCAGAACATCCCAACAGG-3'	5'-GGCGAAGGTACACACAACCA-3'
Calcitonin gene-related polypeptide alfa (alpha-CGRP)	BC028771	5'-AAGTTCTCCCCTTTCCTGGTTGTC-3'	5'-TGGTGGGAACAA AGTTGTCCTTCAC-3'
Calcitonin gene-related polypeptide receptor (CGRP-RCP)	AF118271	5'-CTGGGCAGCAGAACTTGAACGCC-3'	5'-GAGAGAGGGGGTCAGGC-3'
Transforming growth factor β1 (TGFβ1)	NM_011577	5'-CACCATCCATGACATGAACC-3'	5'-TCATGTTGGACAACTGCTCC-3'
Nerve growth factor (NGF)	M35075	5'-GCCTCAAGCCAGTGAAATTAGG-3'	5'-ACGACCACAGGCCAAAACTC-3'
Vascular endothelial growth factor (VEGF)	M95200	5'-CACTGGACCCTGGCTTTACTGC-3'	5'-CGCCTTGGCTTGTCAC-3'
Epidermal growth factor (EGF)	NM_010113	5'-CCAAACGCCGAAGACTTATCC-3'	5'-TGATCCTCAAACACGGCTAGAGA-3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	BC110311	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-CCTGCTTCACCACCTTCTTGA-3'
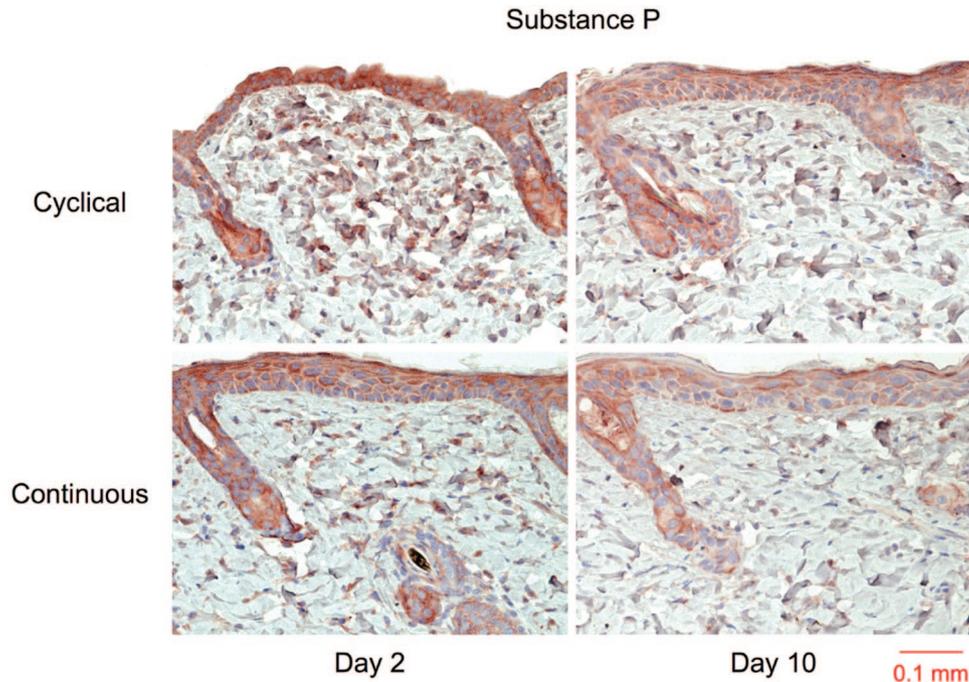
Fig. 4. In the epidermis and dermis, both cyclical and continuous stimulation resulted in a significant increase in accumulation/expression of substance P at 2 days, returning to baseline at 10 days after stretch.
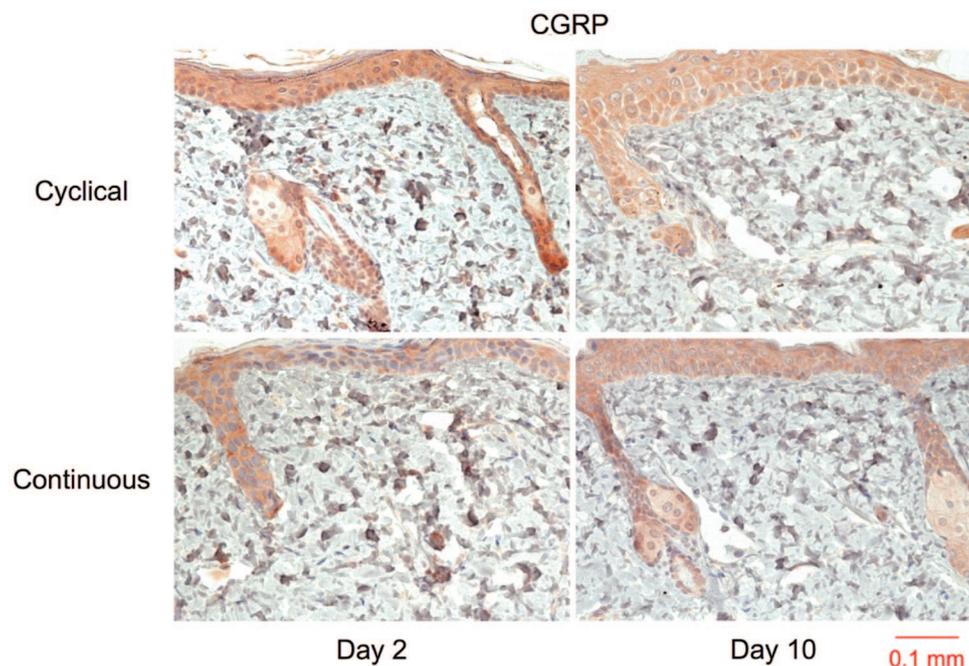
Fig. 5. In the epidermis and dermis, both cyclical and continuous stimulation resulted in a significant increase in accumulation/expression of calcitonin gene-related peptide (*CGRP*) at 2 days, returning to baseline at 10 days after stretch.

cyclical stretch led to significantly more expression of substance P, calcitonin gene-related peptide, NGF, and TGFβ1 than continuous stretch at 2 days. In the results of quantitative analysis (Fig. 8), both cyclical and continuous stretch led to significantly increasing numbers of cells positive for substance P, calcitonin gene-related peptide, TGFβ1, and NGF cells compared with internal

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TGFβ1
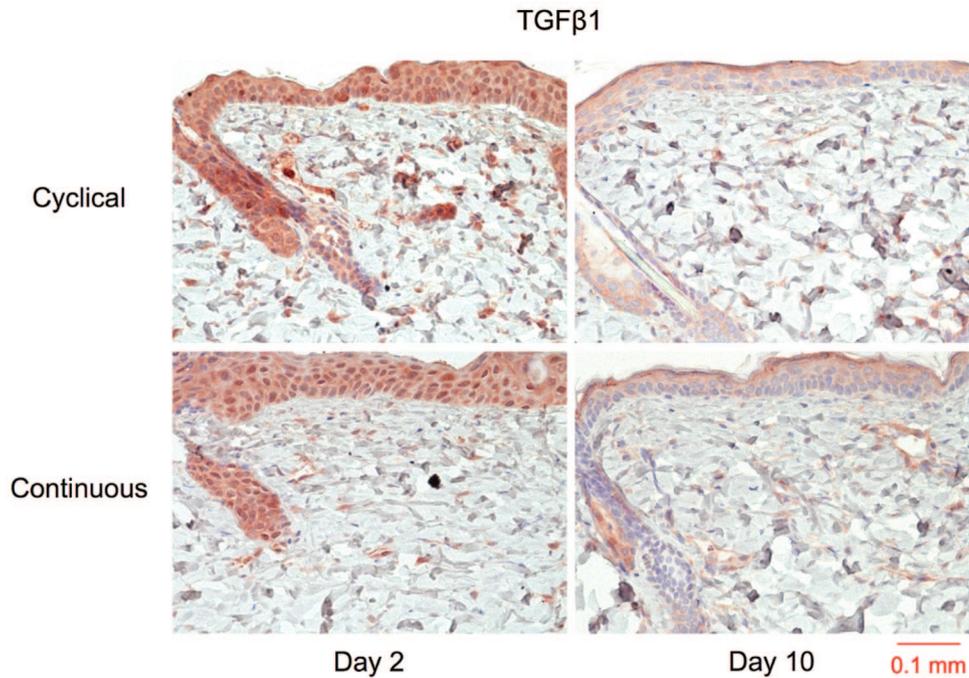
Fig. 6. In the epidermis and dermis, both cyclical and continuous stimulation resulted in a significant increase in accumulation/expression of transforming growth factor β1 (*TGFβ1*) at 2 days, returning to baseline at 10 days after stretch.

NGF
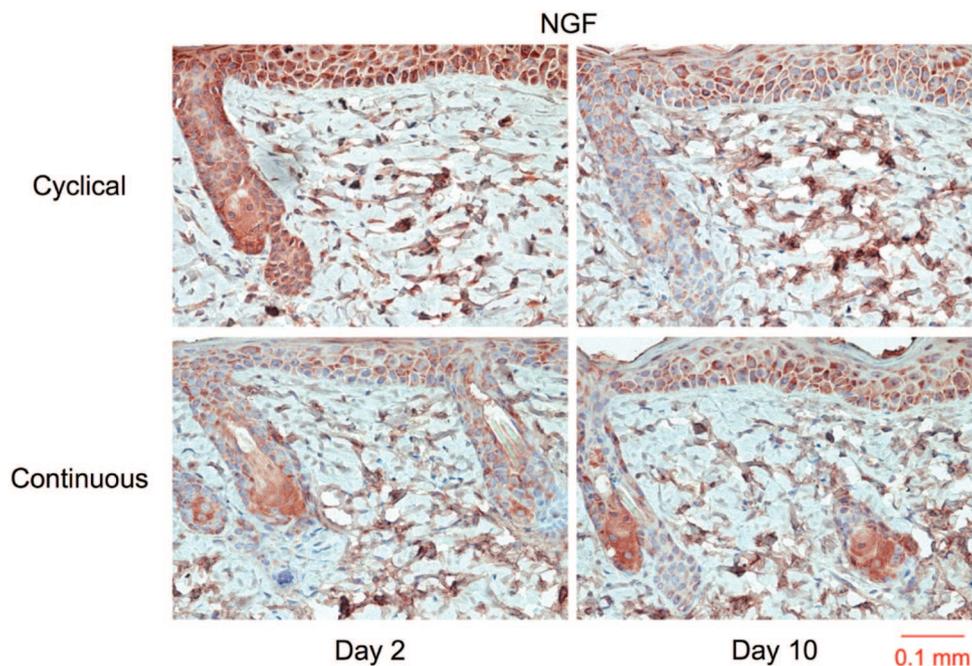
Fig. 7. In the epidermis and dermis, both cyclical and continuous stimulation resulted in a significant increase in accumulation/expression of nerve growth factor (*NGF*) at 2 days, returning to baseline at 10 days after stretch.

tissue controls at 2 days ($p < 0.01$) except TGFβ1 after continuous stretch. At 10 days after stretch, that of substance P and NGF still had a significant difference between cyclical stretched skin and control skins ($p < 0.01$). With regard to continuous stretched skin at day 10, TGFβ1 and NGF had a significant difference ($p < 0.05$). When comparing stretching patterns (Fig. 9), cyclical stretch

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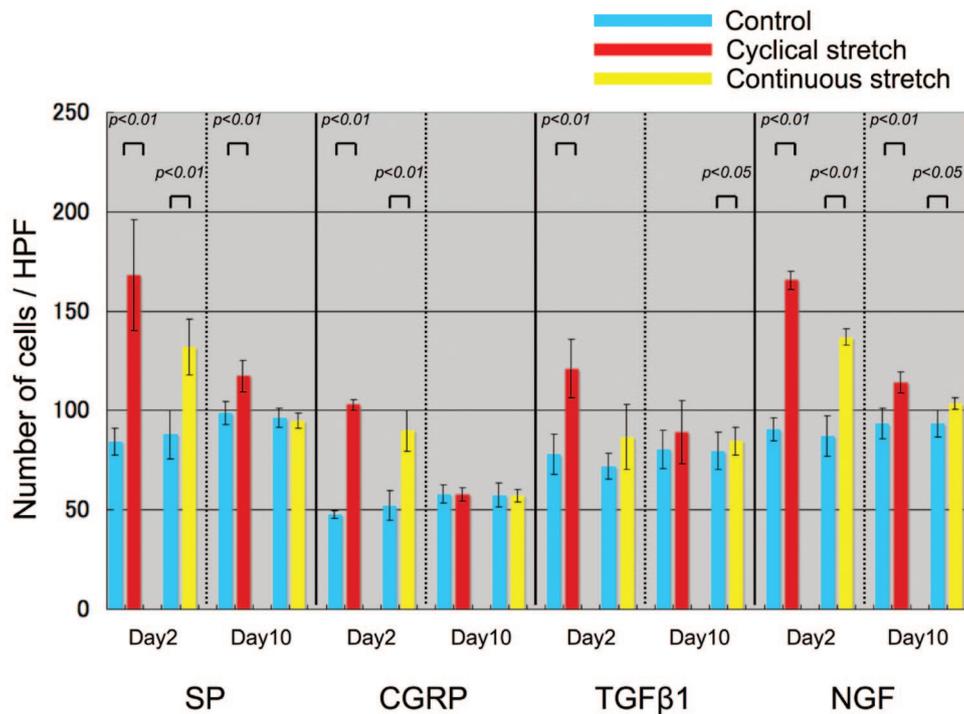
Fig. 8. Quantitative analysis of immunohistochemistry (internal control skin versus cyclical/continuous stretched skin). Five samples in each group ($n = 20$) were used for this study. With the exception of TGFβ1 after continuous stretch, both cyclical and continuous stretch led to significantly greater numbers of cells positive for substance P (*SP*), calcitonin gene-related peptide (*CGRP*), TGFβ1, and NGF compared with internal tissue controls at 2 days ($p < 0.01$). At 10 days after stretch, there was still significant difference in substance P and NGF between cyclical stretched and control skins ($p < 0.01$). With regard to continuous stretched skin at day 10, TGFβ1 and NGF had a significant difference ($p < 0.05$).
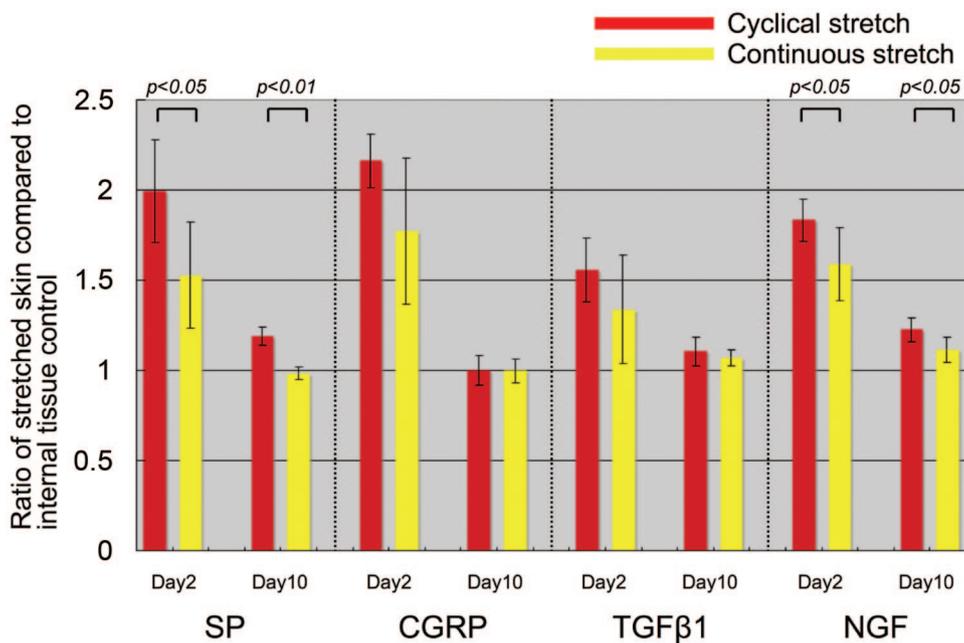
Fig. 9. Quantitative analysis of immunohistochemistry (cyclically versus continuously stretched skin). The numbers of cells positive for substance P (*SP*) and NGF of cyclical stretch were significantly higher than those of continuous stretch. *CGRP*, calcitonin gene-related peptide.

elevated substance P 2-fold from baseline, whereas continuous stretch led to only a 1.5-fold increase ($p < 0.05$). Similarly, relative NGF elevation in cyclical stretch was significantly greater when compared with continuous stretch.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Gene expression of neuropeptides (Tac1 and α-calcitonin gene-related peptide) and growth factors (TGFβ1, NGF, VEGF, and EGF) in cyclical stretched skin was significantly up-regulated with 95 percent confidence compared with their internal tissue controls in relative quantification (Fig. 10). Moreover, receptors of neuropeptides (NK1R and calcitonin gene-related peptide receptor component protein) were significantly down-regulated after cyclical stretching. Significant genomic expression change in continuous stretched skin was observed only with NGF and EGF.

DISCUSSION

Neurogenic Inflammation of Skin

Neurogenic inflammation is defined as cutaneous antidromic vasodilatation and plasma ex-travasation. It is mediated by the release of neuropeptides from sensory endings and skin cells, and it has been suggested that mechanical forces stimulate neuropeptide production and neurogenic inflammation. Neuropeptides are expressed in the skin and often contact epidermal and dermal cells, directly modulating the functions of skin resident cells.[14,15] Among these neuropeptides, substance P and calcitonin gene-related peptide act through neurokinin 1 receptor and calcitonin gene-related peptide receptor (calcitonin gene-related peptide receptor component protein), respectively; substance P and calcitonin gene-related peptide are synthesized under the regulation of NGF.[6,16]

Clinically, neuropeptide activity in skin inflammation can be observed in the form of erythema, edema, hyperthermia, or pruritus.[18] Neurogenic inflammation is closely associated with psoriasis[19,20] and atopic dermatitis,[20,21] and may also be associated with growth and/or generation of abnormal scarring including keloids and hypertrophic scars.[4,22] Scott et al.[23,24] reported increased concentrations of substance P in burn wounds and hypertrophic scars and showed that
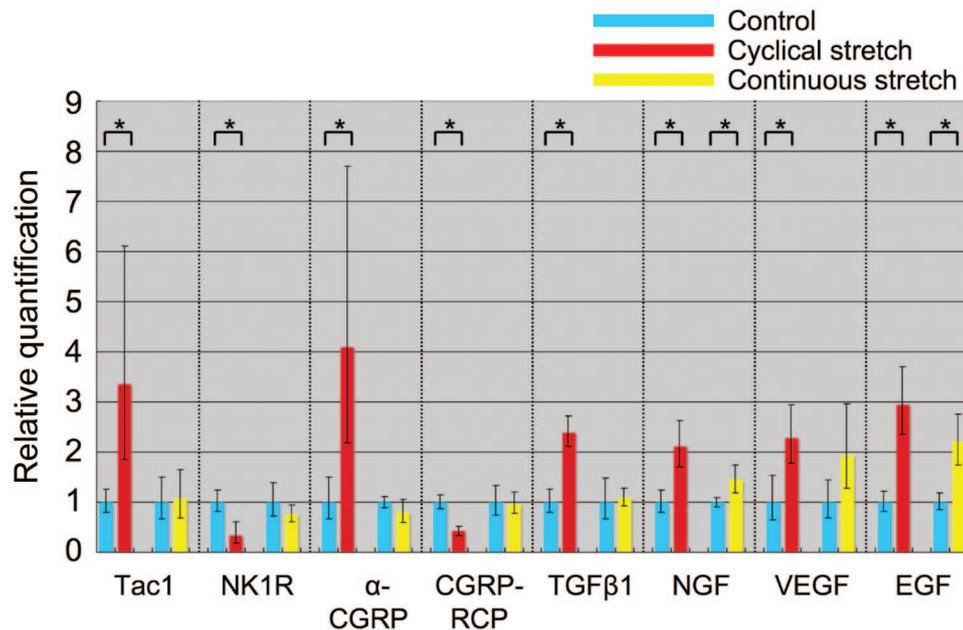
Fig. 10. Real-time reverse-transcriptase polymerase chain reaction. Three tissue samples in each group ($n = 12$) were used for this study. Gene expression of neuropeptides [Tac1 and α-calcitonin gene-related peptide (α-CGRP)] and growth factors (TGFβ1, NGF, VEGF, and EGF) in cyclical stretched skin was significantly up-regulated compared with their internal tissue controls in relative quantification (RQ). Moreover, receptors of neuropeptides [NK1R and calcitonin gene-related peptide receptor component protein (CGRP-RCP)] were significantly down-regulated after cyclical stretching. Continuously stretched skin only demonstrated significant genomic expression change in NGF and EGF.

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topical substance P increases inflammatory cell density in genetically diabetic murine wounds.[25] These studies suggest that neuropeptides are closely related to cutaneous wound healing and scarring.

Neurogenic Inflammation Caused by Skin Stretching

Mechanical force, including skin stretching, may stimulate mechanosensitive nociceptors on sensory fibers or resident cells (Fig. 11), but further studies are needed to demonstrate the effects of mechanotransduction in resident cells. Our study showed that accumulation of neuropeptides in both epidermis and dermis were significantly increased after cyclical stretching of skin. Real-time reverse-transcriptase polymerase chain reaction shows local up-regulation of neuropeptide mRNA gene transcription in the skin, which proves that neuropeptides are released from not only terminal nerve fibers but also resident skin cells. In our study, the expression of the corresponding receptors was significantly down-regulated, which suggests that negative feedback may have occurred in response to the accumulation of excess neuropeptides. Further work will be required to clarify the cell type(s) associated with neuropeptide expression.

Growth factors, including VEGF, EGF, TGFβ1, and NGF, were also significantly elevated after skin stretching. These results suggest that angiogenesis, epithelialization, and collagen production may be associated with neurogenic inflammation.

In our study, we did not observe histamine and central sensitization, but future studies hold promise for the development of treatment for itch
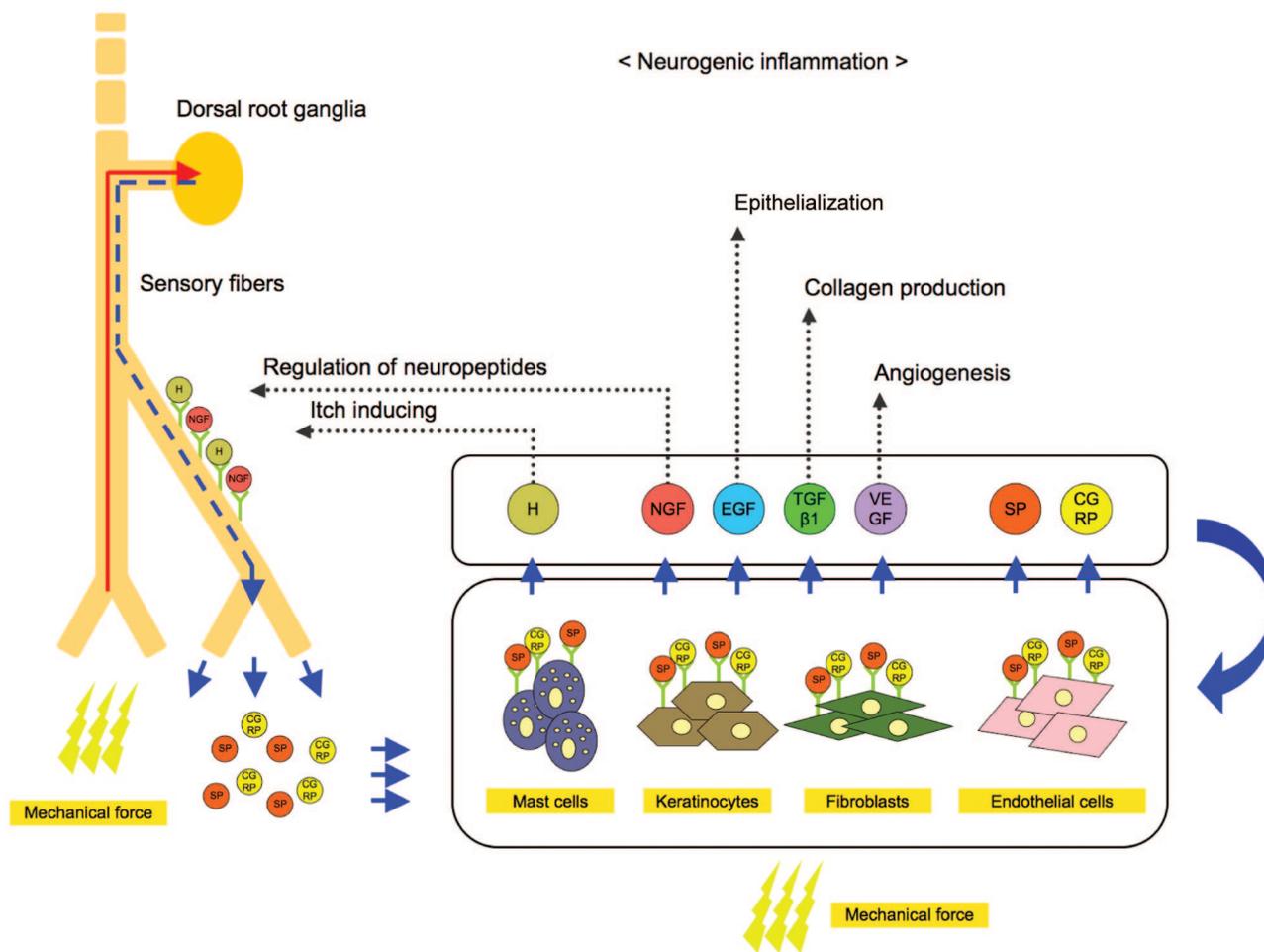
Fig. 11. Schema for select effects of skin stretching and neurogenic inflammation. In our study, neuropeptide accumulation/expression in the skin was up-regulated. We propose that skin stretching may stimulate skin resident cells or mechanosensitive nociceptors in the skin. Several growth factors and chemical mediators may be involved in this "neurogenic inflammation." *H*, histamine; *NGF*, nerve growth factor; *EGF*, epithelial growth factor; *TGFβ1*, transforming growth factor β1; *VEGF*, vascular endothelial growth factor; *SP*, substance P; *CGRP*, calcitonin gene-related peptide.

and pain associated with skin diseases, wounds, and abnormal scarring. With respect to the secretion of histamine from mast cells evoked by neuropeptides, histamine may conjugate with H1 receptors on vascular smooth muscle cells, which would also cause vasodilation and permeabilization of vessels.[26,27] In addition, histamine conjugated with H1 receptors on C fibers, causing itching. Itch histamine-sensitive mechanosensitive purinoceptors and probably mechanosensitive purinoceptors transmit the response to the spinal cord. Noxious input in the spinal cord can induce central sensitization for pain, and purinoceptive input can provoke central sensitization for itch.

Characteristics of Waveform-Specific Skin Stretching

Our results show that cyclical skin stretching is a much stronger stimulus of neuropeptides and growth factor expression in the skin when compared with continuous stretching. Cyclical mechanical force may more effectively stimulate tissues and cells. In theory, two structural cellular elements are involved in mechanical stimulation of skin: mechanosensitive nociceptors and mechanoreceptors on cells. It is well known that pain is increased after cyclical electrical nerve stimulation; however, the direct effect of cyclical force on cells has not been well studied. Various structures have been suggested to function as mechanoreceptors, including mechanosensitive channels,[28–30] the cytoskeleton (particularly the actin filaments), and cell adhesion structures on the cell membrane such as the desmosome. It has been suggested that mechanosensitive channel activation changes the cell membrane potential and induces the influx of Ca^{2+}.[28–30] Thus, cyclical skin stretch may alter cell physiology more effectively than continuous stretch through mechanoreceptors. In addition, mechanical signal transduction pathways related to skin stretching such as extracellular signal-regulated kinase pathways[31] and phosphoinositide 3-kinase pathways[32] should also be studied in our model in the future.

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