Short communication

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NERVE GROWTH FACTOR INJECTED INTO THE GASTRIC ULCER BASE INCORPORATES INTO ENDOTHELIAL, NEURONAL, GLIAL AND EPITHELIAL CELLS: IMPLICATIONS FOR ANGIOGENESIS, MUCOSAL REGENERATION AND ULCER HEALING

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A previous study has demonstrated that locally administered growth factors such as epidermal growth factor, basic fibroblast growth factor and hepatocyte growth factor can accelerate healing of experimental gastric ulcers (GU) in rats (1). That study showed that such locally administered growth factors can exert potent biological effects resulting in enhanced gastric ulcers healing. However, the fate of injected growth factors, their retention and localization to specific cellular compartments have not been examined. In our preliminary study, we demonstrated that local injection of nerve growth factor to the base of experimental gastric ulcers dramatically accelerates ulcer healing, increases angiogenesis - new blood vessel formation, and improves the quality of vascular and epithelial regeneration. Before embarking on larger, definitive and time sequence studies, we wished to determine whether locally injected nerve growth factor is retained in gastric ulcer’s tissues and taken up by specific cells during gastric ulcer healing. Gastric ulcers were induced in anesthetized rats by local application of acetic acid using standard methods; and, 60 min later fluorescein isothiocyanate-labeled nerve growth factor was injected locally to the ulcer base. Rats were euthanized 2, 5 and 10 days later. Gastric specimens were obtained and processed for histology. Unstained paraffin sections were examined under a fluorescence microscope, and the incorporation of fluorescein isothiocyanate-labeled nerve growth factor into various gastric tissue cells was determined and quantified. In addition, we performed immunostaining for S100β protein that is expressed in neural components. Five and ten days after ulcer induction labeled nerve growth factor (injected to the gastric ulcer base) was incorporated into endothelial cells of blood vessels, neuronal, glial and epithelial cells, myofibroblasts and muscle cells. This study demonstrates for the first time that during gastric ulcer healing locally administered exogenous nerve growth factor is retained in gastric tissue and is taken up by endothelial, neural, muscle and epithelial cells. This is likely the basis for the therapeutic action of locally administered nerve growth factor and its stimulation of angiogenesis, tissue regeneration and gastric ulcer healing.

Key words: nerve growth factor, tyrosine receptor kinase A, gastric ulcer, angiogenesis, gastric healing
Rats were anesthetized and after laparotomy acetic acid (80 µL) was applied to the serosa of glandular stomach at the anterior wall through a polyethylene tube (6.0 mm inner diameter) for 90 seconds to induce GUs as described previously (1, 2). Sixty minutes later 200 µL PBS (control) or fluorescein isothiocyanate (FITC)-labeled NGF (100 µg/kg body weight in 200 µL PBS) was injected into four quadrants of the submucosa at the GU base (50 µl of the solution per one quadrant), similar to our previous study (2). There were 2 rats each in control and 2 day ulcer groups, 5 rats in the 5 day ulcer group, and 4 rats in the 10 day ulcer group. Two, 5 or 10 days after GU induction, rats were euthanized using carbon dioxide gas, and gastric tissues were collected. The GUs together with a specimen of adjacent non-ulcerated gastric tissue were excised, and divided into two equal parts by cutting through the middle of the ulcer. One half of the ulcer specimen was embedded in O.T.C compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen and stored at –80°C; the remaining half of the ulcer specimen was fixed in 10% buffered formalin and processed for histology. Frozen sections were stained with DAPI as described in a previous paper (3) and examined using a fluorescence microscope with dual red and green filters (Olympus, Tokyo, Japan). Unstained histologic sections of formalin fixed specimens were also examined by fluorescence microscopy under fluorescence Zeiss Axioimager microscope (Carl Zeiss Microscopy, NY). Separate sections were immunostained for S100β protein using specific antibody (Santa Cruz Biotechnology, CA), Alexa 568 (red) labeled anti-mouse, and Alexa 488 (green) labeled anti-rabbit. The sections were counterstained with DAPI (blue) and examined using dual blue and green filters (Carl Zeiss Microscopy, NY).

Labeling of nerve growth factor with fluorescein isothiocyanate

Rat NGF protein (N2513; Sigma-Aldrich St. Louis, MO) was labeled with FITC using a commercial kit (Pierce-Thermo Fisher Scientific, Rockford, IL) according to manufacturer’s instructions. The principle of labelling is that the N-hydroxysuccinimide (NHS)-ester labeling reagent (NHS-FITC) reacts efficiently with primary amino groups (-NH₂) such as lysine side chains within the protein to be labeled and forms stable amide bonds.

Immunostaining for S100β protein

The S100β protein is known to localize to the neural elements, e.g. astrocytes, Schwann’s cells, ependymomas, and astrogliomas and in Langerhans cells in the skin. We performed immunostaining using a specific S100β antibody (1:200 mouse monoclonal, Santa Cruz Biotechnology, CA), Alexa 568 (red) labeled anti-mouse, and methods described in our previous studies (2, 4, 5) to determine whether the injected labeled NGF is localized to the neural components during GU healing. The fluorescence signal intensity (FSI) of injected labeled NGF and the S100β staining intensity were quantified using MetaMorph 7.0 (Molecular Devices, PA).

Results

Retention and localization of locally injected FITC-labeled NGF into ulcerated gastric tissue

Gastric sections of control rats injected with PBS did not show any fluorescence. In contrast, in rats injected locally with FITC-labeled NGF, green fluorescence was retained in gastric tissue at 5 and 10 days after its injection. FITC-labeled NGF was localized to endothelial cells of blood vessels and myofibroblasts in granulation tissue, neuronal and glial cells of the submucosa adjacent to GUs, muscle cells of muscularis propria adjacent to the GU, and also to some regenerating gastric epithelial cells at the ulcer base (Figs. 1-3). Interestingly, endothelial cells of numerous blood vessels, both those fully developed as well as those forming endothelial tubes and undergoing angiogenesis showed a strong fluorescence reflecting an uptake and incorporation of FITC-labeled NGF to these cells (Figs. 1A, 1B, 2F; 3A).

Quantification of fluorescence signal intensity showed that uptake of labeled NGF was the strongest in the neural component (glial cells, neuronal cells and nerve fibers) and in endothelial cells, and muscle cells (Table 1). FITC-labeled NGF was also present in

**Fig. 1.** Visualization of locally injected FITC-labeled NGF and DAPI staining in various cells during GU healing at 5 days. FITC-labeled NGF injected at the site of GU induction is retained in gastric tissue, is taken up, and localizes to: (A) endothelial cells of blood vessels (red arrow) and neuronal cells (yellow arrows); (B) budding endothelial cells forming new blood vessels in granulation tissue (pink arrow), and neuronal cells (yellow arrows); and (C) neuronal cells (yellow arrows) nerve fibers (yellow arrowheads) and glial cell (red arrow).
some epithelial cells (Fig. 2D); however, the signal intensity was 2.4-fold lower (P < 0.001) vs. neural or endothelial cells.

DISCUSSION

Our previous studies demonstrated that locally administered FITC-labeled antibodies to survivin and EGF are retained in esophageal, gastric mucosal and in pancreatic tissues, despite histologic processing (6, 7). Our more recent studies demonstrated expression of NGF and its TrkA receptor within the gastric mucosa, primarily in neural elements, endothelial cells of blood vessels and epithelial cells (4).

The present study showed for the first time that FITC-labeled NGF injected into the GU site is retained in the GU granulation tissue and/or ulcer margin, and localizes to the

Fig. 2. Five days after local injection at the site of GU induction, labeled NGF is retained in gastric tissue and localizes to: (A) neuronal cells (yellow arrows), nerve fibers (yellow arrowhead), and glial cells (red arrow); (B) nerve fibers (yellow arrowheads) and glial cells (red arrows); (C) muscle cells (MC) and nerve fibers (yellow arrowheads); (D) epithelial cells (white arrowheads) of regenerating mucosa; (E) glial cells (red arrows) and myofibroblasts (red arrowhead); and (F) endothelial cells of blood vessels (pink arrows).

Fig. 3. Ten days after local injection at the site of GU induction. (A) FITC-labeled NGF is retained in gastric tissue and localizes to: neuronal cells (yellow arrows), nerve fibers (yellow arrowheads), glial cells (red arrows), endothelial cells of blood vessels (pink arrows) and epithelial cells (white arrowheads). (B) S100β expression (red staining) is localized mainly to neuronal cells, nerve fibers and glial cells.
uptake was blocked by specific inhibitors of TrkA and p75NTR. Red-labeled NGF and I\(^{125}\) NGF binds to its receptors tyrosine receptor kinase A, high affinity tropomyosin related kinase (TrkA) receptor and a high affinity NGF receptor (8, 9). The biological actions of NGF are mediated through two types of specific receptors with distinct affinities - a high affinity tropomyosin related kinase (TrkA) receptor and a low affinity receptor, p75NTR (16, 17). Previous studies focused on neuronal cells showed an uptake of fluorescent dextran-Texas Red labeled NGF and \(^{125}\)I-labeled NGF by neural cells and this uptake was blocked by specific inhibitors of TrkA and p75NTR receptors (8, 9).

NGF has been shown to exert actions on other non-neuronal cells and increased expression of NGF has been demonstrated in response to acute tissue injury (18-20), indicating its active role in response to injury and in initiation of injury healing. Exogenous NGF has been shown to accelerate healing of injured muscles when given by intramuscular injection, and of corneal ulcers when administered topically (20, 21). The precise mechanisms underlying tissue injury healing action of NGF are not known, but potentially may involve activation of other growth factors (e.g., VEGF), and stimulation of neovascularization (15, 22, 23).

Recent studies provide evidence of NGF's possible involvement in angiogenesis. NGF has been shown to induce in vitro proliferation of HUVECs and brain capillary endothelial cells (14, 24) and can upregulate VEGF production in some cells (19, 25). The effect of NGF on angiogenesis in gastric endothelial cells has not been examined previously except our preliminary studies (26), which showed that NGF stimulates angiogenesis in gastric endothelial cells (26). From that study we can infer that the NGF injected into gastric tissue and taken up by endothelial cells can directly stimulate in vivo angiogenesis in GU granulation tissue.

Emmanueui et al. demonstrated that NGF injected to the muscle of ischemic limbs promotes reparative neovascularization and reduces endothelial and muscle cell apoptosis (20). Our present study demonstrating that locally injected NGF localizes to the endothelial cells of blood vessels and muscle cells provides a mechanistic explanation for those findings and a basis for tissue injury healing actions of NGF.

Table 1. Quantification of FITC-labeled NGF taken up and incorporated into various cellular components of GUs. Fluorescent staining intensity (FSI) was determined in 10 standardized mucosal sections using MetaMorph 7.0 videomage system software. P value when compared to neuronal cells.

<table>
<thead>
<tr>
<th>Cellular Components</th>
<th>FSI</th>
<th>Fold change</th>
<th>P value*</th>
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<tbody>
<tr>
<td>Endothelial cells</td>
<td>153.02 ± 15.80</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Glial cells</td>
<td>176.92 ± 11.25</td>
<td>1.14</td>
<td></td>
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<tr>
<td>Neuronal cells</td>
<td>155.46 ± 15.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve fibers</td>
<td>153.14 ± 26.99</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Muscle cells</td>
<td>131.97 ± 12.74</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>65.43 ± 19.92</td>
<td>2.38</td>
<td>&lt; 0.001</td>
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endothelial cells of blood vessels and myofibroblasts in granulation tissue, to the neuronal and glial cells and nerves fibers of the submucosa, the muscle cells of muscularis propria; and, to some regenerating gastric epithelial cells at the ulcer base. Interestingly, numerous newly formed vessels at this early stage of gastric ulcer healing consisted of endothelial tubes and showed strong staining reflecting incorporation of FITC-labeled NGF into endothelial cells undergoing angiogenesis.

The mechanism(s) of NGF incorporation into the above cells within the GU is not known, but one might expect that injected NGF binds to its receptors tyrosine receptor kinase A, high affinity NGF receptor (TrkA) and/or p75NTR on cells expressing them. NGF was discovered as a peptide factor crucial for growth and survival of neurons (10-13). NGF is produced by neuronal cells and in some tissues by fibroblasts, epithelial cells and ECs (14, 15). The biological actions of NGF are mediated through two types of specific receptors with distinct affinities - a high affinity tropomyosin related kinase (TrkA) receptor and a low affinity receptor, p75NTR (16, 17). Previous studies focused on neuronal cells showed an uptake of fluorescent dextran-Texas Red labeled NGF and \(^{125}\)I-labeled NGF by neural cells and this uptake was blocked by specific inhibitors of TrkA and p75NTR receptors (8, 9).

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Gastric mucosa, submucosa and muscularis propria are densely innervated by enteric neurons that form the enteric nervous system and by primary afferent neurons that arise from cell bodies in either dorsal roots (spinal afferent) or are vagal afferents (27-31). Enteric neurons and enteric glial cells may modulate epithelial cells functions, which is the basis for an emerging concept of a digestive “neuronal-glial-epithelial unit” similar to the neuronal-glial-endothelial unit in the brain (32). Enteric glial cells are no longer viewed as just being the essential “glue” of the enteric nervous system and have been shown to also be neuronal precursors (33-35). Enteric glial cells can be experimentally induced to give rise to neurons (33-35).

This study demonstrated for the first time that locally injected NGF into the GU base is retained in gastric tissue in the ulcer area and incorporates into the endothelial cells of blood vessels undergoing angiogenesis. NGF was also incorporated into myofibroblasts in granulation tissue, the neuronal and glial cells and nerves fibers of the submucosa, the muscle cells of muscularis propria and to some regenerating gastric epithelial cells at the ulcer base. Thus, this study provides the basis for the future use of local NGF injection (via an endoscope) for the treatment of chronic, non-healing gastric ulcers in humans and possibly other tissue injuries.

Abbreviations: bFGF, basic fibroblast growth factor; EC, endothelial cell; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; GU, gastric ulcer; HGF, hepatocyte growth factor; NGF, nerve growth factor; P75NTR, low affinity NGF receptor; TrkA, tyrosine receptor kinase A, high affinity NGF receptor.

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