Review Article

Roles of calcitonin gene-related peptide in ehancement of angiogenesis

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Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide produced by tissue-specific alternative splicing of the primary transcript of the calcitonin/CGRP gene. CGRP is widely distributed in the central and peripheral neuronal systems and exhibits numerous biological activities in mammals. Recently, it was clarified that endogenous CGRP released from primary afferent neurons facilitates the neovascularization indispensable to tumor growth. Angiogenesis in surgical sponge models that mimics tumor stromal reaction was inhibited by a CGRP antagonist, CGRP8-37. The unilateral sciatic nerves ($L_{1\text{-}5}$) of mice were cut, and Lewis lung carcinoma (LLC) cells were implanted into the subcutaneous tissues of the legs. Tumor growth was significantly reduced in the sites of denervation of these mice, compared with the sham-operated mice. In CGRP knockout mice, the tumor growth and tumor-associated angiogenesis of implanted LLC cells were significantly reduced compared with those in wild-type mice. In LLC-bearing wild-type mice, CGRP precursor mRNA levels in the dorsal root ganglion were increased compared with those in non-LLC bearing mice. This increase was abolished by denervation. In a co-culture system using human umbilical vein endothelial cells (HUVEC) and fibroblasts, CGRP increased tube formation by endothelial cells. VEGF expression was up-regulated in the tumor implantation models in a CGRP-dependent manner. These recent results suggest that sensory nerves facilitate tumor-associated angiogenesis and tumor growth during tumor development via a release of CGRP. The same was true in the processes of wound healing and gastric ulcer healing. Further, CGRP was also reported to enhance the angiogenesis in hind-limb ischemia model. These recent results indicate CGRP may become a novel therapeutic target for controlling angiogenesis.

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Functional and morphological relationships between primary afferent neurons and blood vessels

Functional and morphological relationships between primary afferent neurons and blood vessels during cancer development are poorly understood. However, recent evidence suggests that physical and biochemical interactions between these peripheral components are important to both tumor biology and cancer-associated pain [1]. Postnatal neovascularization occurs through (i) capillary sprouting of resident endothelial cells (EC) as angiogenesis, (ii) proliferation of pre-existing arteriolar connections, a process named arteriogenesis, and (iii) *de novo* vascularization from EC precursors, that is, vasculogenesis, a process thought to be peculiar to the embryo, but possibly observable also in adults [2, 3]. It is widely known that the neuronal system plays a fundamental role in the maturation of primitive embryonic vasculature. Mutations that disrupt peripheral sensory nerves or Schwann cells prevent proper arteriogenesis, while those that disorganize the nerves maintain the alignment of arteries with misrouted axons [4]. It has also been reported that sensory neurons modulate the expression of arterial markers on ECs *via* the secretion of vascular endothelial growth factor (VEGF) 164/120. These data suggest that, during development, peripheral nerves have a role in determining the organ-specific patterns of blood vessel branching and arterial maturation [5]. Furthermore, some neuronal factors such as Notch and neuropeptide Y were reported to have roles in tumor-associated angiogenesis [6, 7].

CGRP

CGRP, a 37-amino acid neuropeptide, has various biological actions, including responses to sensory stimuli, cardiovascular regulation, and vasodilation [8]. Recent studies using genetically engineered mice have shown that CGRP-knockout mice exhibit increased blood pressure [9]. CGRP is synthesized by sensory C-fibers throughout the respiratory tree, and potently constricts airway smooth muscle [10]. The tissues contained considerably more CGRP than another neuropeptide, substance P, CGRP [11, 12]. We previously reported that CGRP also exhibited important roles in the gastrointestinal tract [13, 14]. Maintenance of gastric mucosal integrity is highly dependent on the alarm systems which can rapidly sense the harmful chemical or mechanical stimuli to which the mucosa is exposed. The gastrointestinal tract is known to be rich in neuronal systems, among which afferent neurons of extrinsic origin are reported to operate as an emergency protective system [15]. The functions of these afferents sensitive to chemicals are reported to be mediated by CGRP released in the gastric mucosa [15, 16]. It was previously reported that the neuropeptide substance P had a proangiogenic activity [17], although the details of the mechanism of this proangiogenic activity were not studied precisely. CGRP receptor signaling links the activation of adnylate cyclase, suggesting that CGRP may up-regulate VEGF expressions. To confirm the proangiogenic activity of CGRP in vivo, the tube formation assay was tested using human umbilical vein endothelial cells (HUVECs) co-cultured with fibroblasts [18]. When HUVECs were cultured in the presence of CGRP, substantial formation of tube structure positive to CD31 antibody was observed [18]. These suggested that CGRP exhibited proangiogenic activities, and CGRP released from sensory nerves may become a regulator of angiogenesis in pathological conditions.

Roles of CGRP in enhancement of tumor-associated angiogenesis

Pain is the most disruptive influence on the quality of life of cancer patients [1]. Primary afferent sensory neurons exhibit a significant role in which sensory information from peripheral tissues is transmitted to the spinal cord and brain. The cell bodies of the sensory nerve fibers that innervate the head and body are located in the trigeminal ganglia and dorsal root ganglia, and can be divided into two main categories: myelinated A-fibers and thinner, unmyelinated C-fibers. Thin sensory fibers, unmyelinated C-fibers and finely myelinated A-fibers, Aδ, are specialized sensory neurons known as nociceptors, the main function of which is to detect environmental stimuli. Nociceptors express a diverse repertoire of receptors and transduction molecules that can sense forms of noxious stimulation (thermal, mechanical and chemical) with varying degrees of sensitivity [17].

In addition to cancer cells, tumours consist of stromal tissues including recruited hemaotopoietic inflammatory cells, blood vessels, and adjacent primary afferent nociceptors. Cancer cells and stromal cells release a variety of products, such as ATP, bradykinin, H+, nerve growth factor, prostaglandins

and vascular endothelial growth factor (VEGF), that either excite or sensitize the nociceptor [1]. Painful stimuli are detected by the nociceptors, the cell bodies of which lie in the dorsal root ganglion (DRG), and are transmitted to neurons in the spinal cord. The signal is then transmitted to the higher centers of the brain. Nociceptor activation results in the release of neurotransmitters, such as calcitonin gene-related peptide (CGRP), endothelin, histamine, glutamate and substance P [1]. These sensory nerve-derived mediators have vasodilating actions, and as a result of these, the blood supply to the tumor tissues may be increased.

In the recent study, it was tested whether or not CGRP could enhance angiogenesis in vivo. Further, since tumor-associated angiogenesis may be enhanced when sensory nerves were stimulated, it was also tested whether CGRP released as a result of axon reflex could enhance tumor-associated angiogenesis. To test these issues, a strain of knockout mice in which the genes for CGRP are disrupted (CGRP-/-) was developed [9]. It was tested that endogenous CGRP enhanced tumor-associated angiogenesis when tumor pain is introduced.

When Lewis lung carcinoma (LLC) cells were implanted subcutaneously to wild type mice (WT), gradual tumor growth was observed [18]. Subcutaneous infusion of a CGRP antagonist, CGRP8-37, with mini-osmotic pumps suppressed tumor growth. Histological examination revealed that neovascularization was predominantly observed in the stroma of the implanted tumors, and that that in CGRP antagonist-treated mice was suppressed compared with vehicle-treated WT. To block release of the CGRP delivered by axonal transport from the peripheral neurones, the sciatic nerves at the distal parts from the sciatic dorsal ganglions were cut in WT. When LLC cells were implanted subcutaneously at the denervated site, growth of the tumor in the denervated site was suppressed, compared with that in the sham-operated site. Neovascularization in denervated WT was suppressed, compared with that in the sham-operated WT.

When the mRNA levels of proCGRP, a precursor of CGRP, in dorsal root ganglions ($L_{1\text{-}5}$) were determined in tumor-bearing WT, tumor implantation to the area innervated by $L_{1\text{-}5}$ resulted in the increased expression of proCGRP in sham-operated WT, in comparison with that in sham-operated WT without tumor implantation [18]. By contrast, when sensory nerves were cut at the distal site of DRGs, tumor implantation did not increase the expression of proCGRP in dorsal root ganglions even with LLC tumors [18]. These findings suggested that tumor implantation up-regulated proCGRP in the DRGs innervating the area of implantation.

Immunohistochemical localization of VEGF shows that the expression of VEGF in the surrounding stromal tissues was markedly suppressed in CGRP knockout mice, although that in tumor cells was not different between WT and CGRP knockout mice [18]. These results taken together suggested that the downstream molecule relevant to CGRP-dependent enhancement of angiogenesis is VEGF.

Roles of CGRP in enhancement of angiogenesis during development of chronic inflammation and healing processes of wounds and gastric ulcers

It was reported that the chronic proliferative inflammation observed around the sponge implants was also regulated by endogenous CGRP derived from the neuronal systems [18]. VEGF was important as a down stream molecule of CGRP receptor signaling. The same was true in the healing processes of skin wounds and gastric ulcer healing [19, 20]. Healing of ulcer elicited by acetic acid in CGRP-/- was markedly delayed, compared with that in WT. In WT, granulation tissues were formed at the base of ulcers, and substantial neovascularization was induced, whereas those were poor in CGRP-/- [20]. Expression of VEGF was more markedly reduced in CGRP-/- than in WT [20]. The same machinery was active in the skin wound healing process [19]. CGRP has a preventive action on gastric mucosal injury and a proangiogenic activity to enhance ulcer healing.

Roles of CGRP in enhancement of angiogenesis during recovery from ischemia

It is known that a major symptom of arteriosclerosis obliterans is a pain, which may induce the release of CGRP. Since the neural system plays a fundamental role in neovascularization, CGRP released during pain of arteriosclerosis obliterans may have a role in angiogenesis during ischemia. A recent study examined whether endogenous CGRP released from neuronal systems facilitates revascularization in response to ischemia using CGRP-/- [21]. CGRP-/- or their WT littermates were subjected to unilateral hindlimb ischemia. CGRP-/- exhibited impaired blood flow recovery from ischemia and decreased

capillary density expressed in terms of the number of CD-31-positive cells in the ischemic tissues compared with WT. In vivo microscopic studies showed that the functional capillary density in CGRP-/- was reduced. Hindlimb ischemia increased the expression of pro-CGRP mRNA and of CGRP protein in the lumbar dorsal root ganglia. Lack of CGRP decreased mRNA expression of growth factors, including CD31, vascular endothelial growth factor-A, basic fibroblast growth factor, and transforming growth factor-β, in the ischemic limb tissue. The application of CGRP enhanced the mRNA expression of CD31 and VEGF-A in HUVECs and fibroblasts. Subcutaneous infusion of CGRP8-37, a CGRP antagonist, using miniosmotic pumps delayed angiogenesis and reduced the expression of proangiogenic growth factors during hindlimb ischemia. These suggested that endogenous CGRP facilitates angiogenesis in response to ischemia. Targeting CGRP may provide a promising approach for controlling angiogenesis related to pathophysiological conditions.

Perspective

Cancer pain is a critical determinant of the patients's QOL. The negative impact that cancer pain has on QOL cannot be overestimated. As advances in cancer detection and therapy are extending the life expectancy of cancer patients, there is an increasing focus on improving patients' quality of life. For many patients, pain is the first sign of cancer, and 30–50% of all cancer patients will experience moderate to severe pain [22]. Cancer can cause pain at any time during its course, but the frequency and intensity of pain tend to increase during the advanced stages. In fact, 75–95% of patients with metastatic or advanced-stage cancer will experience significant amounts of cancer-induced pain [23]. Neuronal system-derived CGRP was believed to promote tumor growth by vasodilation to increase the supply of nutrients for tumors. But, as described in this review article, CGRP can increase the density of the newly formed blood vessels as a result of angiogenesis. This may be a novel activity of the neuropeptide CGRP. Recent developments of CGRP antagonists are highly expected to use in the treatments of migraine [24]. As discussed, CGRP together with neuronal system blockade may become a novel therapeutic target for cancers, however, we have to pay attentions to the possible adverse effects such as delay in healing of wounds and ulcers together with reduced recovery from the ischemia.

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