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Cyclooxygenase Expression in Canines Following Peripheral Nerve Injury

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Abstract In order to investigate the mechanism of neurogenic pain, this study used a median nerve compression model in dogs. The nerve was compressed with a clip for three weeks. Immunohistochemistry was done by the avidin-biotin-peroxidase complex method to observe the changes of T cells (CD45) and macrophages (Mac-1) after compression. Antibodies against cyclooxygenase (COX)-1 and 2 were used to examine the localization and changes of these mediators caused by nerve compression. In control animals, resident T cells were detected, but there were no macrophages. COX-2 was positive in the Schwann cells and vascular endothelial cells, while COX-1 was detected in the vascular endothelial cells. After nerve compression, numerous T cells and macrophages appeared among the demyelinized nerve fibers. The macrophages were positive for COX-2. COX-2 may be deeply involved in neuritis arising from mechanical compression, and this mediator seems to be important in the manifestation of neurogenic pain.

Keywords: *peripheral nerve, wallerian degeneration, macrophage, cyclooxigenase, entrapment neuropathy*

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1. Introduction

The main cause of neurogenic pain is considered to be inflammatory changes resulting from compression of the nerve by surrounding tissues such as bone and cartilage, with inflammatory cells, and prostaglandin (PG) being assumed to play an important role. Cyclooxygenase-2 (COX-2) has been recently cloned and it is an inducible isomer of cyclooxygenase (COX), the enzyme that produces PG involved in the development of pain [1]. COX-2 is found in a variety of tissues, and it is suggested to play an essential role in the development of inflammatory pain via the production of PG [2]. However, many points remain unknown with regard to the roles played by COX in the development of neurogenic pain in patients with entrapment neuropathy, such as carpal tunnel syndrome and cubital tunnel syndrome. In order to investigate the mechanism of neurogenic pain in these diseases, this study used a median nerve compression model in dogs. Immunohistochemistry was used to examine the localization and changes of inflammatory cells and COX.

2. Materials and Methods

2.1. Production of Nerve Compression Model

The experiment was carried out under the control of the local animal ethics committee in accordance with the guidelines on animal experiments in our university, Japanese government animal protection and management law, and Japanese government notification on feeding and safekeeping of animals. Twelve adult mongrel dogs weighing 7-15 kg were used in the study. Under anesthesia, the skin of the palmar aspect of the distal 1/3 of the foreleg was cut, and the median nerve was exposed and entrapped with a clip for microvascular suturing (Kouno Co, Chiba, Japan). In the present study, the strength of the spring clips used for nerve compression was determined with an Instron-type tensile tester (AGH-2000B, Simazu Co., Kyoto, Japan). Based on the assumption that the strength of the springs follows the law of Young, the weight required to open a clip by 2 mm was determined and five types of clips with different compression forces were prepared by adjusting the strength of the spring. The compression force required to open a clip by 2 mm was determined because the longitudinal diameter of the median nerve is 3.5 ± 0.3 mm on average (n=10). The pressure actually applied to the nerve can be calculated in mmHg from the following equation: 1033.6 gram force /cm^2= 760 mmHg. The area (cm^2) of a clip in contact with the nerve is 0.2 cm (width

of a clip) x 0.35 cm (longitudinal diameter of nerve) = 0.07 (cm^2). Therefore, 1 g of force represents about 8.75mmHg of pressure. The median nerve was clamped at 7.5 gram force (approximately 65 mmHg) clipping power [3]. The contralateral median nerve was also exposed. However, the contralateral median nerve that was not entrapped but was used as a sham. After the clip was applied to the median nerve, the wound was closed and the animal models were sacrificed after either 1 or 3 weeks.

2.2. Histological and Immunohistochemical Analysis

After the appropriate period of nerve compression, the animals were fixed by intra-aortal 4% paraformaldehyde perfusion. The specimens were embedded in paraffin and stained with hematoxylin and eosin (HE). Immunohistochemical staining was done by the avidin-biotinperoxidase complex (Vectastain ABC kit, Vector Laboratory Inc., CA). The primary antibodies were anti-human T cell (CD45, 1: 10dilution, M742, DakoCytomation Denmark A/S, Glostrup, Denmark) as a T cell marker and anti-mouse CD11b/18 (Mac-1; Ly40, 1:10 dilution, CL8941AP, Ceder Lane, Ontario, Canada) as a marker of macrophages. Further, anti-cyclooxygenase-1 (COX-1 [C-20]: 1:100 dilution, cat # sc-1752, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and anti-cyclooxygenase- 2 (COX-2 [C-20], 1:100 dilution, cat # sc-1745, Santa Cruz Biotechnology) were used to examine intraneural COX.

The number of positive cells per unit square were compared between the non-compressed and compressed nerves. A total of 40 serial cross-sections were prepared that covered the compressed part of each nerve. The number of positive cells was quantified by a real-time image analysis system (LUZEX FT, Nikon, Tokyo, Japan) under a light microscope with a 100X objective. The intraneural cells have a diameter of about 5-10 μm, so 5-μm thick sections were observed every 4th section to avoid counting the same cells more than once. A total of 10 sections of each nerve were observed to count the number of positive cells per 0.5 mm^2, which was then multiplied by 4 to obtain the number of cells per unit square. The mean number of positive cells per unit square was compared between the non-compressed and compressed nerves.

2.3. Statistical Analysis

Significant differences of prior comparison was determined using a one-way ANOVA with repeated measures and post hoc (Scheffe) compared before compression of nerve. Date were entered into a database and analyzed by using SPSS statistical software, version 14.0.J (SPSS Inc., Chicago, IL). A probability of 5% was considered statistically significant.

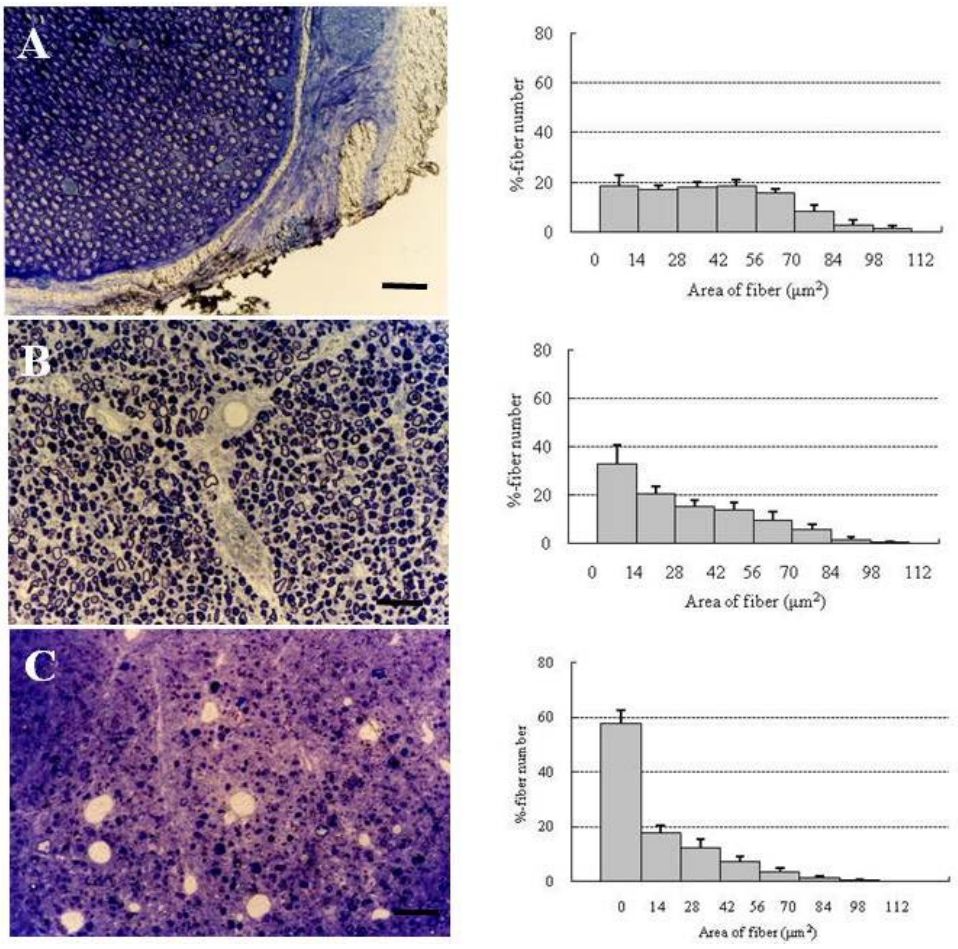

Figure 1. Light micrographs and nerve fiber morphology before (A) and after compression(B,C). (Toluidin blue stain: original magnification. X50, bar: 100μm): (A) Control group. No Wallerian degeneration was evident in the nerve. (B) After 1 weeks of compression, a fall out in the large myelinated fiber population at the periphery of the nerve root is noted. (C) After 3 weeks, nerve fiber degeneration was more advanced than after 1 week. In morphometric fiber analysis, the fibers in the fascicles demonstrated a preponderance of smaller myelinated fibers in one (B) or three weeks (C) of compression

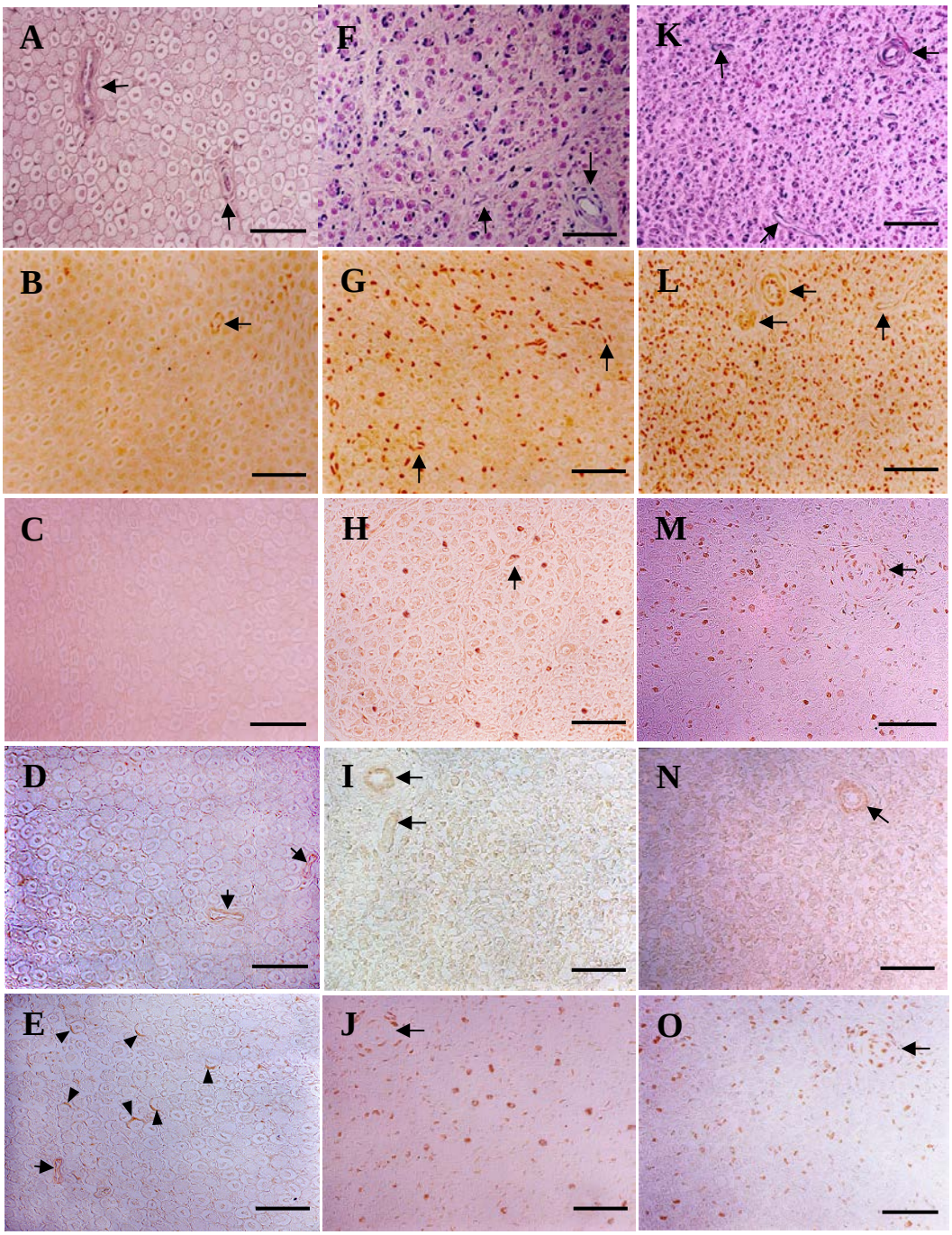

Figure 2. Histological (H&E stain) (A,F,K) and immunohistochemical changes after nerve compression. (A-E) In the normal state (control), (F-J) 1-week compression group, (K-O) 3-weeks compression group. (Original magnification: X100, bar: 100μm). (A,F,K) H&E stain. In the normal state, no Wallerian degeneration was evident in the nerve (A). Intraneural Wallerian degeneration was observed one and three weeks after compression (F, K). (B,G,L) CD45-positive T cells. In the normal state, some CD45-positive resident T cells appeared in the nerve (B). There was an appreciable increase in the number of cells positive for the T-cell marker CD45 as nerve fiber degeneration progressed after 1(G) and 3 weeks (L). T cells were invaded in the endoneural space from the blood vessels (G,L: arrows). (C,H,M) Mac-1-positive macrophage. In the normal state, Mac-1-positive macrophage didn't show in the nerve (C). After nerve compression, some Mac-1 positive macrophages appeared in the endoneural space from the blood vessels (H,M: arrow) and increased in areas affected by Wallerian degeneration with time. (D,I,N) COX-1-positive cells. In the normal (D) and pathological state (I, N), expression of COX-1 was limited to vascular endothelial cells (arrows) (D). (E,J,O) COX-2-positive cells. In the normal state, COX-2 expression was apparent in vascular endothelium (arrows) and Schwann cells (arrowheads) (E). After nerve compression, however, the number of COX-2-positive macrophages was invaded in the endoneural space from the blood vessels (arrows) (J) and markedly increased significantly by compression after 3 weeks (M,O)

3. Results

Histological examination revealed no degeneration of the nerve fibers in the control group (Figure 1A, Figure 2A). The nerve contained many myelinated nerve fibers as well as occasional collagen fibers and blood vessels in the endoneurial spaces among the nerve fibers. In the immunohistochemically stained specimens some CD45-positive resident T cells were seen in the nerve (Figure 2B), but not Mac-1 positive macrophages (Figure 2C). COX-1 (Figure 2D) and COX-2 (Figure 2E) were positive in vascular endothelial cells alone, or in Schwann cells and vascular endothelial cells, respectively.

After 1 week of compression, the nerve contained many demyelinated nerve fibers, and numerous inflammatory cells were seen among the separated nerve fibers (Figure 1B, Figure 2F). In morphometric fiber analysis, the fibers in the fascicles demonstrated a preponderance of smaller myelinated fibers in one or more weeks of compression. In the immunostained sections, large numbers of T cells (Figure 2G) and macrophages (Figure 2H) were observed between the degenerating nerve fibers, chiefly at the site of compression. Using Mac-1-positive sections, the existence of COX-2 in the macrophages could be confirmed (Figure 2J). Numerous macrophages were also observed in areas affected by Wallerian degeneration peripheral to the compressed site in the nerve. However, no increase in COX-1 positive cells was seen when compared with the control group (Figure 2I). The relative number of small-diameter axons increased at 3 weeks after compression compared with those in the 1-week compression group (Figure 1C, Figure 2K). T cells (Figure 2L) and macrophage (Figure 2M) numbers became even more marked 3 weeks after compression. The macrophages were positive for COX-2 (Figure 2O). On the other hand, no increase in COX-1 positive cells was seen when compared with the control group (Figure 2N). The results of ANOVA and Scheffe test showed the increase of CD45 after 1 week and 3 weeks compression ($F=225.84$, $P<0.05$.) (Figure 3). The number of Mac-1 and COX-2 increased significantly by compression after 1 and 3 weeks ($P<.0.05$)

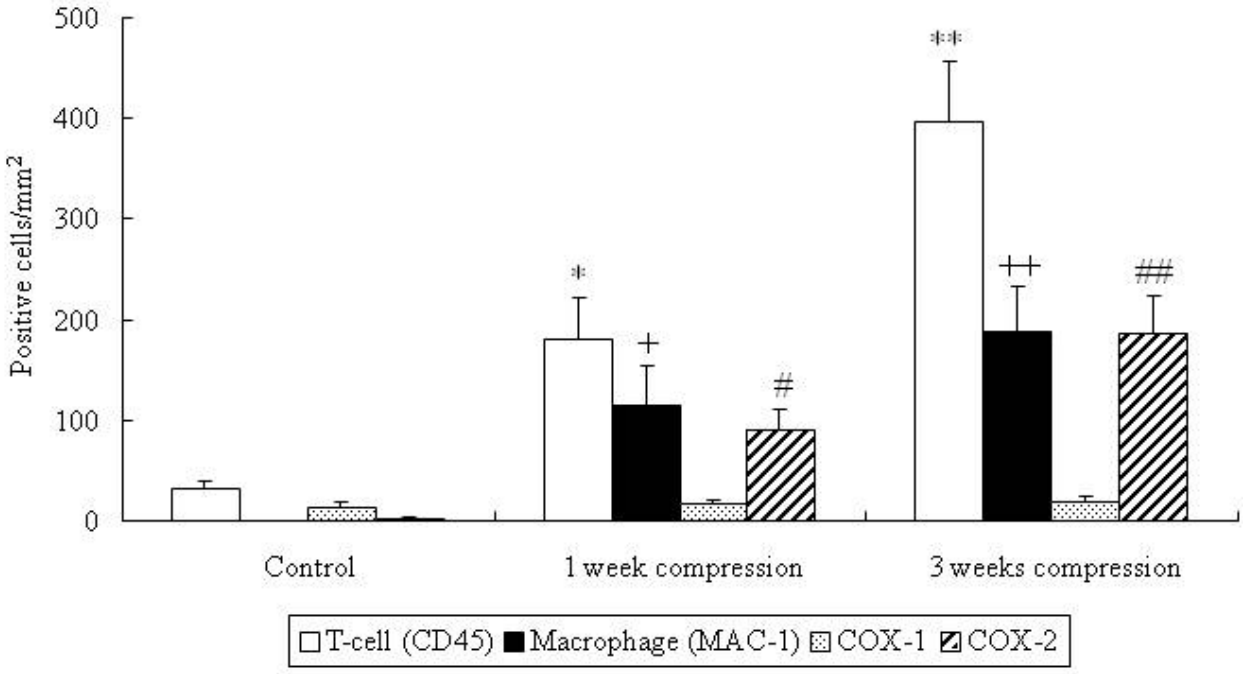

Figure 3. Changes in CD 45, Mac-1, COX-1 and COX-2 positive cells in the nerve after nerve compression. (*, +, #: $p < 0.05$ by Scheffe test for control group vs. 1-week compression group. **, ++, ##: $p < 0.05$ by Scheffe test for control group vs. 3-week compression group. $, $$: p = n.s. by Scheffe test for control group vs. 1-week compression or 3-week compression.)

4. Discussion

Pain is one of the defense mechanisms that enable organisms avert damage. It arises from physical stimulation of nociceptors and from chemical stimulation, especially by the mediators produced at sites of inflammation. Inflammation as one of the defense reactions is aimed at removal of foreign materials and restoration of damaged tissues, among other actions. There is a close relationship between inflammatory changes in nerves resulting from compression and pain manifestation. In this study, the median nerve was clamped at 7.5 gram force (approximately 65 mmHg) clipping power. During nerve compression, there is injury to the nerve fibers and blood flow due to the increase in vascular permeability and the breakdown of the blood-nerve barrier in the intraneural vessels [3], thereby increasing the endoneural pressure and exacerbating the damage to the nerve fibers. Rydevik and Lundborg [4] showed that it is possible to analyze the intraneural blood flow of epineurial vessels and endoneural capillaries during and after localized compression using intravital microscopic techniques. When the nerve was compressed experimentally, external pressures of 20 to 30 mmHg induced a retardation of venular blood flow in the epineurium. Compression at 60 to 80 mmHg induced complete circulatory arrest in the compressed nerve segments. They also showed the leakage of EBA from intraneural microvessels after acute compression [5]. This resulted in edema formation in the endoneurial space occurring at the edges of the compressed segments in all fascicles as the nerves were compressed at 200 and 400 mmHg for 2 hours.

Within the nerve, T cells sense the breakdown of the myelin sheath produced by demyelination or the loss of substances from the blood vessels and release macrophage-activating factors such as interferon-γ (IFN-γ) [6]. Activated macrophages then begin to infiltrate locally, and are thought to phagocytose and remove the necrotic tissue. At the same time, these cells produce and release inflammatory cytokines such as interleukin 1β (IL-1β) [7-13], tumor necrosis factor-α (TNF-α) [14,15] and nitric oxide [13,16-21] that cause neuritis by precipitating neuropathic pain.

Prostaglandins display an important role in inflammatory reactions by reducing the pain threshold of sensory nerve endings [2]. COX is a regulatory enzyme in the biosynthesis of PG, which exhibit diverse physiological activities, or thromboxanes. COX is a two-step enzyme, i.e., it first oxidizes arachidonic acid by its oxygenase activity to synthesize PGG_2, and then reduces PGG_2 by its peroxygenase activity to synthesize PGH_2. This enzyme is a glycoprotein of about 70 kDa, which forms a homodimer to show activity. COX is a hemoprotein, and its subunits each contain one molecule of iron (III)-protoporphyrin [22]. It had been confirmed that there are two COX isozymes: COX-1 is a constitutive enzyme produced by a housekeeping gene, while COX-2 is an inducible enzyme produced by an immediate early gene. COX-1 remains active at a constant level throughout the cell cycle. It is found in almost all tissues and it is locally involved in a wide variety of biological responses. On the other hand, COX-2 is inactive under normal conditions, but is induced rapidly and transiently by cytokines such as IL-1β and TNF, and then disappears within a few hours. COX-2 is found in macrophages and fibroblasts, and is said to be involved in the production of prostaglandin during inflammation and neovascularization [23,24].

It is very difficult to distinguish acute from chronic compression to the nerve as the cause of neuropathic pain. The nerves always move with movement of the joints, and the dynamic limit is dependent on the positional relationship between the nerve and the surrounding tissues. Talmor M, et al. reported that syovial hypertrophy is a prominent finding in carpal tunnel syndrome [25]. They demonstrated that COX-2 is up-regulated in the tenosynovium of patients with carpal tunnel syndrome, and this upregulation may correlate with the clinical grade of the tenosynovium. It is suggested that the syovial hypertrophy may cause adhesions between the lesion and the nerve, which then may reduced the mobility of the nerve during movement of the joints. This led to severe tension or compression on the nerve [26], thus causing disturbance of intraneural blood flow and breakdown of the blood-nerve barrier, resulting in intrarneural inflammatory changes such as edema and demyelination [3,27]. Thus, when narrowing of the carpal tunnel is caused by synovitis, movement of the nerve along with movement of the wrist and fingers becomes limited, and consequently compression and traction on the nerve cause neuropathic pain. Chronic compression is the result of repeated episodes of mild acute compression. Gupta et al showed that an early consequence of chronic nerve compression is local demyelination and remyelination, which may be the primary cause of alterations in nerve function during the early period post-compression [28]. According to our clinical experience, neuropathic pain is frequently alleviated by rest in patients with carpal tunnel syndrome and is intensified by application of acute compression to the nerve during the Phalen test [29].

From the fact that inflammatory cytokines induce COX-2 in various cells and that selective inhibitors of COX-2 block pain in inflammatory models and clinical trials, it is suggested that COX-2 plays an important role in the manifestation of the inflammatory pain via PG [30,31,32]. Two cited papers showed that abundant COX-2 expressing cell profiles were present in injured sciatic nerve 2 and 4 weeks following partial sciatic nerve ligation and most of these COX-2 expressing cells were identified as macrophages [33,34]. And also, Ma W et al. demonstrated that partial nerve injury induces more abundant COX-2 expression than complete nerve injury [35]. In the present study, we recognized COX-1 and COX-2 in the vascular endothelial cells of normal nerves, while COX-2 was seen in the Schwann cells. After nerve compression, the number of COX-2-positive macrophages was markedly increased significantly by compression after 3 weeks, though COX-1-positive cells showed little change compared with the control group. The compressed region of the nerve also contained numerous macrophages that were positive for COX-2. Macrophages participate in the autoregulatory loop in the inflammatory process. Circulating macrophages are the major effectors cells that invade the degenerating nerve, and these immune cells not only clear up degraded nerve debris but also produce and release pro-inflammatory cytokines, growth factors and pro-nocicetive mediators. Inducible COX-2, which drives PGE_2 production, is also synthesized de novo in macrophages upon proinflammatory stimulation [36,37]. On the other hand, it is apparent from the natural history of nerve regeneration that macrophages essential for clear up degraded nerve debris, although it produces pro-nocicetive mediators, such as IL-1β, TNF-α, i-NOS and COX-2 [13,34,35]. Therefore, control of the inflammatory reaction is an important challenge when treating patients with injured nerve.

Non steroidal anti-inflammatory drugs (NSAIDs) are most frequently used in the treatment of neuropathic pain. The activity of COX is inhibited by drugs such as aspirin, ibuprofen, and indomethacin. The analgesic effect and anti-inflammatory action of these drugs is considered to be based on the inhibition of COX-2, while adverse effects such as gastrointestinal and renal disorders are thought to occur because constitutive COX-1 is inhibited [24]. It is therefore anticipated that the next generation of anti-inflammatory drugs for neuropathic pain will be selective COX-2 inhibitors that can be used without such side effects.

Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgments

The submitted manuscript does not contain information about medical devices or drugs. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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