3. DISCUSSION

3.1. The controversial role of granulocytes in the generation of inflammatory pain

Leukocytes contain mediators that have properties to cause or to ameliorate pain. Several previous studies have examined whether granulocytes cause pain. In these studies granulocytes were recruited using local injections of fMLP, leukotriene B4, complement C5a, and nerve growth factor. All of these induced pain in response to mechanical or thermal stimuli that was attenuated by systemic granulocyte depletion (Levine et al., 1984; Levine et al., 1985; Levine et al., 1986; Bennett et al., 1998). However, the results are controversial for three reasons: First, the corresponding receptors of those mediators (FPR for fMLP, C5a receptor, BLT1 for leukotriene B4, trkA for NGF) are not selectively expressed on granulocytes but also on other leukocyte subpopulations such as macrophages as well as on peripheral neurons (Susaki et al., 1996; Yang et al., 2000; Goodarzi et al., 2003). Second, the agents used for granulocyte depletion were nonselective and depleted other leukocyte subpopulations (e.g. macrophages) at the same time (Bennett et al., 1998; Foster et al., 2002). Third, significant differences in nociceptive thresholds were only detected at a time point when both granulocytes and monocytes/macrophages were both significantly depleted (Bennett et al., 1998; Foster et al., 2002). Taken together, a central role for granulocytes in the generation of inflammatory pain has not been conclusively demonstrated in those studies.

In contrast, local injection of CXCR2 ligands resulted in selective recruitment of granulocytes in our studies (Rittner et al., 2006b). CXCR2 ligand inoculation did not induce pain or expression of c-fos in the spinal cord. In contrast, CFA-induced inflammation resulted in pain while the same number of infiltrating granulocytes was recruited. A similar activation state of recruited granulocytes was seen in CFA inflammation and after intraplantar injection of CXCR2 ligands. In other experimental models of peritoneal pain (acid-induced writhing) glycogen-induced granulocyte recruitment even reduced visceral pain (Giorgi et al., 1998). Glycogen-induced granulocyte recruitment is mediated by enhanced local production of CXCR2 ligands (CXCL2/3 and CXCL1) and it could be blocked by anti-CXCR2 ligand antibodies (Mulligan et al., 1993; Remick et al., 2001). Pain in this model was mediated by mast cells and resident macrophages (Ribeiro et al., 2000). In CFA-induced local inflammation, neither granulocyte depletion nor enhancement of granulocyte recruitment altered the intensity of inflammatory pain (Brack et al., 2004a; Brack et al., 2004c; Rittner et al., 2006a; Rittner et al., 2006b). In support of our results, local injection of glycogen was shown to selectively recruit granulocytes without causing pain (Levine et al., 1985). Granulocytes, therefore, seem not to contribute to the generation of pain.

Other groups examined whether chemokines by themselves could induce pain. Different groups of chemokines directly activate nociceptors (Oh et al., 2001; Oh et al., 2002; White et al., 2005). Most of these studies employed other chemokines. However, direct local injection of CXCR2 ligands into non-inflamed paws resulted in pain but the doses used were several orders of magnitude lower than ours and selective granulocyte recruitment was not demonstrated (Cunha et al., 1991; Lorenzetti et al.,

2002; Sachs et al., 2002; Cunha et al., 2005). In support of our results, heat-induced neuropeptide release in a skin nerve preparation was augmented by cytokines (i.e. tumor necrosis factor-α, IL-1β and -6) but not by CXCL8 (Opree and Kress, 2000).

In summary, we provide evidence that granulocytes do not cause pain, if selectively recruited into tissue by chemokines. Further studies will be needed to examine the role of other leukocyte subpopulations in the generation of pain. Chemokines selective for certain leukocyte subpopulations might be a useful tool for this purpose.

3.2. Opioid receptors in inflammation

One of the factors increasing the analgesic efficacy of opioids in inflammation is the increased expression of opioid receptors on peripheral nerve terminals. Besides increased anterograde transport of opioid receptors to the periphery increases in mRNA and protein expression were postulated. Indeed, during CFA-induced paw inflammation we observed an upregulation in κ-opioid receptor mRNA in DRG of the inflamed side 12 hr after CFA injection (Puehler et al., 2006), while previously a biphasic upregulation of μ-opioid receptor mRNA and no change in δ-opioid receptor mRNA content was shown (Puehler et al., 2004). These findings indicate that the three opioid receptors are differentially regulated, as has been postulated before (Zhang et al., 1998). Several mechanisms account for this regulation: κ-opioid receptor mRNA expression in DRG seems to be dependent on the local production of IL-1β in the inflamed hindpaw because an IL-1 receptor antagonist reduces the increased expression of κ-opioid receptor mRNA and protein (Jeanjean et al., 1994; Jeanjean et al., 1995; Puehler et al., 2006). In contrast, the early peak of μ-opioid receptor mRNA upregulation in DRG appears to be regulated by electrical conduction because nerve blockade by local anesthetics inhibits this upregulation (Puehler et al., 2004). In DRG μ-opioid receptor expression at later stages of the inflammation is regulated by nerve growth factor (Zwick et al., 2003; Molliver et al., 2005; Mousa et al., 2007b). Local injection of nerve growth factor alone mimics the increased binding of opioid receptor agonists in DRG seen in CFA inflammation. Supporting this mechanism, Mousa et al. showed that local injection of anti-nerve growth factor antibodies reduces CFA-induced upregulation of opioid receptor agonist binding in the DRG. This anti-nerve growth factor treatment also abolished the augmentation of opioid receptor expression on peripheral nerve terminals and of opioid induced analgesia in CFA inflammation. The exact mechanisms of nerve growth factor and IL-1β induced upregulation of opioid receptors remain to be elucidated. In summary, three factors were so far identified to regulate opioid receptor expression IL-1β, nerve growth factor, and electrical conduction.

3.3. Opioid peptides in inflammation

Inflammatory mediators not only regulate opioid receptor expression but also the synthesis of opioid peptides. Full length proopiomelanocortin mRNA as the precursor of β-endorphin is essential because it contains the signal sequence ensuring processing to and secretion of the authentic peptide (Clark et

al., 1990). In the past, several studies detected truncated proopiomelanocortin transcripts in naïve lymphocytes (Lacaze-Masmonteil et al., 1987; Cabot et al., 1997). Only one study showed full-length proopiomelanocortin mRNA containing the signal sequence in human leukocytes (Stephanou et al., 1991). However, under pathological conditions or after mitogen treatment of lymphocytes *in vitro* full-length proopiomelanocortin mRNA can be detected (Lyons and Blalock, 1997). Here we provide evidence that signal sequence-encoding proopiomelanocortin mRNA is expressed under control conditions and is upregulated after paw inflammation in B- and T-lymphocytes from the draining lymph node using higher sensitive nested PCR (Sitte et al., 2007). In accordance with other studies the number of copies was low compared to the pituitary (van Woudenberg et al., 1993). In view of the fact that immunoreactive β-endorphin can be easily detected by radioimmunoassay, immunohistochemistry and flow cytometry, other factors such as the mRNA's half-life and translation efficiency might also be important for enhancing protein/peptide production.

In the inflamed paw itself we quantified the leukocyte subpopulations producing opioid peptides after we developed a method to analyze the number of opioid containing leukocytes by flow cytometry (Rittner et al., 2001). The number of opioid containing leukocytes as well as the β-endorphin content in the paw increased during the course of inflammation. At early time points, opioid peptides were produced by granulocytes while later they were produced by macrophages. Only a low percentage of T-lymphocytes were seen in the inflamed paw. Both subpopulations, granulocytes and macrophages, are part of the innate immune reaction as seen in other models of inflammation (Singer and Clark, 1999; Abbadie et al., 2003; Radhakrishnan et al., 2003). Our selective depletion studies confirmed the functional role *in vivo* of granulocytes (Brack et al., 2004a) and macrophages (Brack et al., 2004b) in the generation of analgesia. Interestingly, local transfer of allogenic granulocytes reconstituted analgesia in rat depleted of granulocytes (Rittner et al., 2006c) pointing towards a prominent role of these cells for peripherally mediated opioid endogenous analgesia in the early phase of inflammation.

Under other conditions lymphocytes seem to be important for release of opioid peptides and analgesia. When rats with CFA paw inflammation were immunosuppressed using cyclosporine A, local intraplantar injection of T-lymphocytes reconstitutes opioid mediated analgesia (Hermanussen et al., 2004). In mice with severe combined immunodeficiency lacking T- and B-lymphocytes visceral pain was enhanced compared to wild type mice (Verma-Gandhu et al., 2006). The transfer of T-lymphocytes reduced pain to the level of the wild type mice. The effect was blocked using the peripheral selective opioid receptor antagonist naloxone-methiodide (Verma-Gandhu et al., 2006).

Similar to the previously known opioid peptides (β-endorphin, met-enkephalin and dynorphin), endomorphin-1 and endomorphin-2 are expressed in all leukocyte subpopulations (macrophage/monocytes, granulocytes and lymphocytes) and are increased in inflamed lymph nodes and inflamed subcutaneous paw tissue (Mousa et al., 2002). To test their functional role we found that exogenous endomorphin-1 and endomorphin-2 injected into the inflamed hindpaw were as effective as β-endorphin, but less so than morphine (Labuz et al., 2006). In addition, endogenously produced endomorphin-1 and endomor-

phin-2 seem to contribute to peripherally mediated opioid analgesia elicited by stress or local injection of CRF. However, effects of either single or combined injections of antibodies against endomorphin-1 and endomorphin-2 were weaker than that of anti-β-endorphin antibody. Thus, it appears that leukocyte-derived endomorphins contribute less than leukocyte-derived β-endorphin to attenuation of inflammatory pain.

In conclusion, we and others found that opioid peptides are expressed in several leukocyte subpopulations and upregulated under inflammatory conditions. *In vivo* these peptides seem to play a functional role in the generation of analgesia.

3.4. Chemokines regulating migration of opioid containing granulocytes

Three CXCR2 ligands are produced in the inflamed hindpaw in early stages of CFA inflammation (Brack et al., 2004a; Brack et al., 2004c). They control the migration of opioid containing leukocytes. In our model one CXCR2 ligand can substitute the function of another CXCR2 ligand because the single blockade of one CXCR2 ligand did not impair migration of opioid containing leukocytes. Even double CXCR2 ligand blockade could not completely abolish the migration of opioid containing leukocytes. Therefore, other CXCR2 ligands like CXCL5 (Chandrasekar et al., 2001) or other chemokine receptor systems (Domachowske et al., 2000) or unrelated pathways including formyl peptides (Levine et al., 1985) or complement (Gerard and Gerard, 1994) might be involved. These will be studied in future projects.

Some chemokines are known for their pain causing properties. In some models, injection of CXCR2 ligands caused pain and blockade of CXCR2 receptors reduced inflammatory pain (Cunha et al., 1991; Cunha et al., 1992; Cunha et al., 2000; Lorenzetti et al., 2002; Cunha et al., 2005). Several factors might contribute to these contradictory results: different agents for inflammation (CFA versus carrageenan), different chemokine doses and different methods to evaluate pain behavior (pain threshold versus pain tolerance). Taken together further studies in other models of inflammation are needed to evaluate the biological role of CXCR2 ligands in the generation and control of inflammatory pain.

3.5. Signal transduction leading to opioid peptide release from granulocytes

Granulocytes contain four types of granules that are produced in sequence during maturation and contain characteristic proteins. Our double immunofluorescence confocal microscopy studies of granulocytes revealed that β-endorphin and met-enkephalin are expressed in MPO- and CD63-expressing, but absent in lactoferrin-, metalloproteinase-9 (MMP-9)- or albumin-positive granules indicating the presence of opioid peptides mainly within primary granules (Rittner et al., 2007). Primary granules are formed early during granulocyte maturation (Borregaard and Cowland, 1997; Cieutat et al., 1998). They also harbor bactericidal contents. Thus, our study suggests that β-endorphin and met-enkephalin are expressed early during granulocyte maturation.

Primary granules are released in response to strong stimuli. *In vitro,* granulocytes have to be treated with cytochalasin B disrupting the actin cytoskeleton to allow for secretion of primary granules (Jog et al., 2007; Rittner et al., 2007). The function of the actin cytoskeleton is to limit the rate and extent of granule exocytosis. This seems to be a protective mechanism that prevents release of destructive enzymes unless neutrophils are appropriately primed. *In vivo*, granulocytes are primed by cytokines like tumor necrosis factor-α or IL-1β in the inflamed tissue.

Granulocytes have to be stimulated with a high concentration of a strong stimulus (e.g. fMLP, CXCL8 or leukotriene B4) in order to achieve release from primary granules (Rittner et al., 2007). They require high intracellular Ca^{2+} concentration for exocytosis, while secondary and tertiary granules are mobilized already by weaker stimulation (Sengelov et al., 1993). We found that opioid peptide release from granulocytes was dependent on release of calcium from intracellular stores, but not from calcium influx into the cell from extracellular sources (Rittner et al., 2006c). Upstream of calcium the Gi/Gβγ protein cascade seems to be activated because release can be inhibited by an inhibitor of the IP_3 receptor. This receptor is activated by IP_3 produced by phospholipase C upon Gβγ activation. A second independent pathway regulating opioid peptide release *in vitro* is the activation of PI3K. PI3K regulates degranulation by CXCL8 or other mediators (Knall et al., 1997; Vocks et al., 2003). Regarding the subclass of PI3K involved other groups provided evidence that leukotriene-induced secretion of primary granules is inhibited in PI3Kγ KO mice (Ito et al., 2002; Laffargue et al., 2002). Selective PI3Kδ inhibitors blocked fMLP- and tumor necrosis factor-α-induced release of primary granules (Sadhu et al., 2003). The PI3K class and subclass engaged in CXCL8-induced opioid peptide release still remains to be elucidated.

Thirdly, p38 MAPK activation is necessary for primary granule release (Mocsai et al., 2000). Accordingly, opioid peptide release was dependent on p38 MAPK but not p42 MAPK in our studies (Rittner et al., 2007). MAPK phosphorylate transcription factors regulating gene expression and other proteins to stimulate NADPH oxidase activity, adhesion, degranulation and chemotaxis (Mocsai et al., 2000; Ward et al., 2000; Kasper et al., 2004; Tuluc et al., 2004). Exocytosis of granules is preceded by a number of steps including disruption of the cytoskeleton, migration to the plasma membrane and fusion with the plasma membrane (Logan et al., 2003). Many details of this process are not completely understood in granulocytes and the steps regulated by p38 MAPK remain to be determined. However, differences in membrane fusion protein composure in different granule subpopulations (Mollinedo et al., 2006) might explain that primary granule exocytosis is regulated by p38 MAPK whereas tertiary granules are unaffected. In summary, subcellular localization of opioid peptides and intracellular signaling pathways for opioid peptide release follow the known pathways of CXCL8 induced degranulation (Knall et al., 1997; Schorr et al., 1999; Hirsch et al., 2000; McNeill et al., 2007).

To evaluate the *in vivo* relevance of our *in vitro* findings chemokines were tested in CFA inflammation. Injection of CXCL2/3, but not CXCL12, into inflamed paw tissue resulted in local mechanical and thermal analgeia dependent on μ- and δ-opioid receptors (Rittner et al., 2006c) and β-endorphin as

well as met-enkephalin (Rittner et al., 2007). In order to confirm the relevance of intracellular Ca^{2+} for opioid peptide release *in vivo* we employed an approach to avoid impairment of sensory nerve functioning by signal cascade inhibitors (Ji et al., 2007). To this end we established a model of *ex vivo* treatment and subsequent adoptive cell transfer. Rats were depleted of granulocytes and local adoptive transfer of granulocytes from allogenic rats was performed. Transfer of granulocytes reconstituted CXCL2/3-induced analgesia. Granulocytes can be transferred to another rat (Briones et al., 2003). Another group has recently shown that transfer of lymphocytes also restores peripherally mediated opioid analgesia in immunosuppressed rats (Hermanussen et al., 2004). We next used this model to study signaling requirements for opioid peptide release *in vivo.* When we treated these allogenic granulocytes *ex vivo* with an intracellular calcium chelator no reconstitution of CXCL2/3-induced analgesia was achieved. This approach necessitates the use of irreversible inhibitors of the signaling cascade, because competitive inhibitors would be washed away before transfer of allogenic cells. This method was therefore not applicable to study the effect of p38 MAPK inhibition *in vivo*, because SB203580 inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket (Lee et al., 1999). Instead, we directly injected SB203580 intraplantarly, which impaired CXCL2/3-induced analgesia by interfering with release of opioid peptides. Presumably because inflammation induces p38 MAPK activation in the soma of C fiber nociceptors responsible for maintenance of inflammatory heat hypersensitivity (Ji et al., 2002), p38 MAPK blockade caused a small analgesic effect.

In summary, we have characterized the subcellular localization of opioid peptides, the signaling pathways of chemokine triggered opioid peptide release from granulocytes and shown their relevance *in vivo*.

3.6. Clinical implications

Peripherally mediated endogenous opioid analgesia is observed in clinical settings: opioid receptors are expressed on peripheral terminals of sensory nerves in human synovia (Stein et al., 1996; Mousa et al., 2007a). They mediate analgesia in patients with various types of pain including chronic rheumatoid arthritis and osteoarthritis, bone pain, after dental, laparoscopic, urinary bladder and knee surgery (Likar et al., 1997; Likar et al., 1998; Likar et al., 1999; Likar et al., 2001; Stein et al., 2003). Opioid peptides are found in human synovial lining cells, mast cells, lymphocytes and macrophages. The prevailing peptides are β-endorphin and met-enkephalin, while only minor amounts of dynorphin are detectable (Stein et al., 1993; Stein et al., 1996; Likar et al., 2007; Mousa et al., 2007a). The interaction of endogenous synovial opioid peptides with peripheral opioid receptors was examined in two studies in patients undergoing knee surgery. (i) Blocking intraarticular opioid receptors by the local administration of naloxone resulted in significantly increased postoperative pain (Stein et al., 1993). These findings suggest that in a stressful (e.g. postoperative) situation, opioids are tonically released within inflamed tissue and activate peripheral opioid receptors to attenuate clinical pain. (ii) Stimulating opioid peptide release by intraarticular CRF application resulted in a significant but short lasting reduction of postoperative pain under both resting and exercise conditions (Likar et al., 2007). Local

injection of naloxone together with CRF reversed this pain reduction under resting conditions. In contrast to the central nervous system, it appears that immune cell-derived opioids do not readily produce cross-tolerance to morphine at peripheral opioid receptors since intraarticular morphine is an equally potent analgesic in patients with and without opioid-producing inflammatory synovial cells (Stein et al., 1996). Thus, it may be interesting to explore the opioid production/release and the migration of opioid-containing leukocytes as possible treatment options (e.g. acupuncture (Sekido et al., 2004)).

The important role of chemokines in the trafficking of opioid-containing cells to injured tissues as well as release of opioid peptides in inflamed tissue indicates that anti-chemokine strategies for the treatment of inflammatory diseases may in fact carry a significant risk to exacerbate pain. Chemokine receptor antagonists are currently under investigation: For example, CCR1 is responsible for leukocyte recruitment into inflamed tissue and expressed in the synovia in patients with rheumatoid arthritis and on monocytes in active lesions in multiple sclerosis. Several companies have CCR1 antagonists currently in clinical trials for treatment of these diseases (White et al., 2005). Antagonists against CCR3 could potentially help in the treatment of allergy and asthma, because CCR3 is involved in the recruitment of eosinophils, basophils, mast cells macrophages, airway epithelial cells and Th2 cells (Erin et al., 2002). Antagonists against CCR5 reduce viral load in AIDS patients (Barber, 2004).

MAPK inhibitors are presently under investigation for the treatment of rheumatoid arthritis, allergy or cancer (Dambach, 2005; Goldstein and Gabriel, 2005). Findings in neuropathic and inflammatory pain in animals have lead to the concept that these inhibitors might also be useful in the treatment of pain in patients (Ji, 2004; Ji et al., 2007). In our study in inflammatory pain we observed an impaired release of opioid peptides with p38 MAPK inhibition. Therefore, interference with this system using MAPK inhibitors in patients should be carefully evaluated regarding their effect on pain.

Because opioid analgesia resulting from neuro-immune interactions occurs in peripheral tissues, it is devoid of central opioid side effects (such as depression of breathing, nausea, sedation, addiction and tolerance). It is also lacking typical side effects produced by cyclooxygenase inhibitors such as gastric erosions, ulcers, bleeding, diarrhea, thromboembolic events and renal toxicity. In the future it would be highly desirable to identify stimulating factors and strategies that selectively attract opioid-producing cells and increase peripheral opioid receptor numbers in damaged tissue. Augmenting the synthesis and/or secretion of opioid peptides and opioid receptor numbers within injured tissue may be accomplished by gene therapy: delivery of proenkephalin, proopiomelanocortin and of μ-opioid receptor cDNAs have been shown to decrease chronic pain and inflammation (Braz et al., 2001; Lu et al., 2002; Xu et al., 2003).

4. SUMMARY

Inflammatory pain is modulated by hyperalgesic and analgesic mediators. Hyperalgesic mediators include protons, cytokines, chemokines, and bradykinin. Some of these are produced by leukocytes at the site of inflammation. Simultaneously, leukocytes are known to produce analgesic mediators. Of these, opioid peptides including β-endorphin, met-enkephalin and endomorphins have been most extensively studied. Opioid peptides are secreted locally following exposure to stress or by local injection of releasing agents. Following release, they bind to opioid receptors on sensory nerve terminals and confer peripherally mediated opioid analgesia.

In this thesis we examined four aims in inflammatory pain induced by complete Freund's adjuvant (CFA) in rats: i) role of granulocytes in the generation of inflammatory pain, ii) impact of inflammation on transcription of opioid receptor genes in the dorsal root ganglion (DRG), iii) peripherally mediated opioid analgesia by endomorphins, and iv) signaling pathways of opioid peptide release from granulocytes.

Granulocytes and pain: Granulocytes can be selectively recruited into peripheral tissue by intraplantar injection of specific chemokines such as CXCL1 or CXCL2/3. This recruitment does not alter thermal or mechanical nociceptive thresholds. Likewise selective depletion of granulocytes in rats with CFA inflammation does not reduce the intensity of inflammatory pain. Granulocytes therefore do not seem to confer pain.

Inflammation and opioid receptor expression: Previous studies demonstrated that inflammation enhances translation, axonal transport and peripheral expression of opioid receptors. To explore possible mechanisms we demonstrated that injection of IL-1β into noninflamed tissue increases expression of κ-opioid receptor mRNA as well as protein in the DRG similar to CFA-induced inflammation. Furthermore, CFA-induced upregulation of opioid receptor transcription was blocked by pretreatment with IL-1 receptor antagonist.

Novel opioid peptides in peripheral analgesia: Not only opioid peptides such as β-endorphin, met-enkephalin and dynorphin but also the newly discovered endomorphin-1 and -2 can elicit potent analgesia in inflammation. Stress- as well as CRF-induced peripherally mediated analgesia was dependent on the release of classic opioid peptides but also on the release of endomorphin-1 and -2.

Signaling pathways of opioid peptide release *in vitro* and *in vivo*: In the early phase of CFA inflammation granulocytes are the major opioid producing infiltrating leukocytes. Opioid peptides are localized in primary granules in granulocytes and released upon stimulation by e.g. chemokines like CXCR1/2 ligands. CXCR1/2 ligands are known to induce granular release from granulocytes activating a cascade involving Gβγ subunits, phospholipase C and inositol 1,4,5-trisphosphate. In parallel, CXCR1/2 ligands activate phosphoinositol-3-kinase (PI3K) and p38 mitogen activated kinase (MAPK). We demonstrate that opioid peptide release is dependent on these pathways. It requires mobilization of Ca^{2+} from intracellular stores, but is independent of extracellular Ca^{2+}. It can be blocked by specific PI3K and p38 MAPK antagonists *in vitro*. *In vivo*, intraplantar injection of CXCR2 ligands elicits

analgesia. This requires intracellular Ca^{2+} mobilization and opioid peptide release *in vitro* and *in vivo* as shown by adoptive transfer experiments.

Taken together, granulocytes contribute to peripherally mediated opioid analgesia in early inflammation while their role in the generation of inflammatory pain seems to be limited. Interference with granulocyte function using e.g. anti-inflammatory treatments like chemokine receptor antagonists or inhibitors of intracellular signaling pathways might inadvertently impair endogenous peripherally mediated opioid analgesia.

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6.1. Original Articles – included

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- **Rittner HL**, Mousa SA, Labuz D, Beschmann K, Schäfer M, Stein C, Brack A
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6.2. Original Articles – not included

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