Neuroscience 132 (2005) 879–892

A QUANTITATIVE *IN SITU* HYBRIDIZATION AND POLYMERASE CHAIN REACTION STUDY OF MICROGLIAL–MACROPHAGE EXPRESSION OF INTERLEUKIN-1β mRNA FOLLOWING PERMANENT MIDDLE CEREBRAL ARTERY OCCLUSION IN MICE

B. H. CLAUSEN, K. L. LAMBERTSEN, M. MELDGAARD AND B. FINSEN*

Medical Biotechnology Center, University of Southern Denmark, Winsloewparken 25, DK-5000 Odense, Denmark

Abstract—Interleukin-1β (IL-1β) is known to play a central role in ischemia-induced brain damage in rodents. In comparison to the rat, however, the available data on the cellular synthesis of IL-1β mRNA and protein in the mouse are very limited. Here, we report on the time profile, the topography and the quantitative, cellular expression of IL-1β mRNA in mice subjected to permanent occlusion of the distal middle cerebral artery (MCA). The *in situ* hybridization analysis showed that IL-1β mRNA was expressed during the first post-surgical hour in a small number of high-expressing macrophage-like cells, located in cortical layers I and II of the future infarct. At 2 h, a significant number of faintly labeled IL-1β mRNA-expressing cells had appeared in the developing peri-infarct, and the number remained constant at 4 h and 6 h, when the hybridization signal began to distribute to the cellular processes. Quantitative PCR performed on whole hemispheres showed a significant 20-fold increase in the relative level of IL-1β mRNA at 12 h and a highly significant 42-fold increase at 24 h, at which time single IL-1β mRNA-expressing cells were supplemented by aggregates and perivascular infiltrates of intensely labeled IL-1β mRNA-expressing cells. Immunohistochemistry and double immunohistochemical stainings in addition to combined *in situ* hybridization, confirmed that the intensely labeled IL-1β mRNA-expressing and IL-1β protein synthesizing cells predominantly were glial fibrillary acidic protein-immunonegative, macrophage associated antigen-1-immunopositive microglia-macrophages. By day 5 there was a dramatic decline in the relative level of IL-1β mRNA in the ischemic hemisphere. In summary, the data provide evidence that permanent occlusion of the distal MCA in mice results in expression of IL-1β mRNA and IL-1β synthesis in spatially and temporally segregated subpopulations of microglia and macrophages. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: cytokines, focal cerebral ischemia, stroke, astrocytes, immunohistochemistry.

*Corresponding author. Tel: +45-6550-3990; fax: +45-6590-3950.
E-mail address: bfinsen@health.sdu.dk (B. Finsen).

Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CAST, Computer Assisted Stereological Test; DAB, diaminobenzidine; EDTA, ethylenediaminetetraacetate; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HPRT1, hypoxanthine phosphoribosyltransferase 1; HRP, horseradish peroxidase; ICE, interleukin-1 converting enzyme; IL-1β, interleukin-1β; IL-1Ra, interleukin-1 receptor antagonist; IL-1RI, interleukin-1 receptor type I; Mac-1, macrophage associated antigen-1; MCA, middle cerebral artery; NBT, nitroblue tetrazolium; PFA, paraformaldehyde; PMN, polymorphonuclear leukocyte; PVDF, polyvinylidene difluoride membrane; RT, room temperature; RT rt-PCR, reverse transcription real-time polymerase chain reaction; Sø-PB, Sørensen buffer; SSC, saline sodium citrate; TBS, Tris-buffered saline; V_{total}, total volume of the infarct.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.01.031

Interleukin-1β (IL-1β) is known to play a key role in ischemia-induced brain damage both in mice (Hara et al., 1997; Schielke et al., 1998; Boutin et al., 2001) and rats (Garcia et al., 1995; Toulmond and Rothwell, 1995; Betz et al., 1996; Mulcahy et al., 2003). IL-1β mRNA and protein are present in low levels in the normal brain (Lechan et al., 1990) and in elevated levels already 15–60 min after an ischemic insult in the rat (Buttini et al., 1994; Davies et al., 1999). Although IL-1β may also serve neurotrophic and neuroprotective functions in the CNS (Rothwell and Strijbos, 1995; Carlson et al., 1999), the focus on IL-1β in relation to cerebral ischemia is on understanding the mechanism of its neurotoxic effects. In addition to its own gene expression (Schindler et al., 1990), IL-1β induces the synthesis of cyclooxygenase-2 and nitric oxide synthase (Collaco-Morales et al., 1996; Iadecola et al., 1997). IL-1β is also known to activate nuclear factor κ/B (Dinarello, 1996) and mitogen-activated protein kinases p38 (Koistinaho and Koistinaho, 2002; Wang et al., 2002) and p44/42 (Skifter et al., 2002), which play a central role in the intracellular signaling in ischemia-induced brain damage (Koistinaho and Koistinaho, 2002).

IL-1β is produced as a 31 kD biological inactive precursor protein, which is converted to the active 17 kD IL-1 protein through specific enzymatic proteolysis by IL-1 converting enzyme (ICE) (Dinarello, 1994; Schielke et al., 1998). IL-1β binds to either of two IL-1 receptors, both of which are expressed in the brain (Parnet et al., 1994; Yabuuchi et al., 1994a). Interleukin-1 receptor type I (IL-1RI) is an 80 kD molecule, which is widely expressed on neurons (French et al., 1999; Yabuuchi et al., 1994a), glia and endothelial cells (Ericsson et al., 1995; Pinteaux et al., 2002; Pousset et al., 2001). Interleukin-1 receptor type II is a 68 kD decoy receptor (Colotta et al., 1993a), which is expressed on neurons and glial cells (French et al., 1999; Pinteaux et al., 2002), but not on endothelial cells (Colotta et al., 1993b). In addition, the co-receptor accessory protein, which is an essential component of the IL-1R1 receptor regulatory mechanism (Cullinan et al., 1998; Greenfeder et al., 1995; Lang et al., 1998), is expressed by microglia and astrocytes (Pinteaux et al., 2002). Recent studies indicate that IL-1β can mediate signal transduction independently of the IL-1RI, suggesting that there are other functional, yet

unknown, IL-1β receptors in the brain (Parker et al., 2002; Touzani et al., 2002).

Administration of the naturally occurring 17 kD IL-1 receptor antagonist (IL-1Ra) or IL-1β neutralizing antibody has been shown to reduce brain damage after focal cerebral ischemia both in the rat (Garcia et al., 1995; Loddick and Rothwell, 1996; Relton et al., 1996; Yamasaki et al., 1995) and in the mouse (Touzani et al., 2002). While there is strong evidence for the stroke-lesioned rat brain that IL-1β mRNA and IL-1β protein primarily is synthesized by microglia and macrophages (Buttini et al., 1994; Davies et al., 1999; Hillhouse et al., 1998; Yabuuchi et al., 1994b), the existing data on the cellular IL-1β synthesis in focal cerebral ischemia in mice are very limited (Hill et al., 1999; Zhang et al., 1998). This is in sharp contrast to the still increasing use of mice and transgenic mouse models in experimental stroke research (Schielke et al., 1998; Boutin et al., 2001; Touzani et al., 2002). The objective of this study therefore was to provide insight in the quantitative cellular and the spatio-temporal expression of IL-1β mRNA, and the synthesis of IL-1β protein in mice following permanent focal cerebral ischemia using reverse transcription real-time polymerase chain reaction, *in situ* hybridization, immunohistochemistry and stereological counting principles.

EXPERIMENTAL PROCEDURES

Animals

The study was performed using age-matched, young adult, male SJL mice (21–26 g; Bomholtgaard A/S, Ry, Denmark). The mice were housed in separate cages and given free access to food and water. Mice used for histological analysis survived for 30 min (n=7), 1 h (n=7), 2 h (n=7), 4 h (n=7), 6 h (n=7), 12 h (n=9), 24 h (n=14) and 5 days (n=13). In addition, 1 sham-operated mouse was included at each time point. The mice used for reverse transcription real-time polymerase chain reaction (RT rt-PCR) analysis had the following post-surgical survival times: 30 min (n=3), 1 h (n=10), 2 h (n=8), 4 h (n=10), 6 h (n=8), 12 h (n=8), 24 h (n=8) and 5 days (n=4). Ten unlesioned mice were included as controls, and additional 25 mice were used for sham-operations: 1 h sham (n=8), 2 h sham (n=6), 4 h sham (n=8), and 6 h sham (n=3). With the exception of the mice with 24 h survival used for the histological analysis, the experimental material originated from the same mice as used in the study by Lambertsen et al. (2005). Four mice with 24 h postsurgical survival time were used for immunohistochemical staining. Care was taken to minimize the number of animals and their suffering. The animal procedures were conducted according to the Danish Animal Health Care Committee and international guidelines on ethical use of animals.

Permanent middle cerebral artery (MCA) occlusion

Focal cerebral ischemia was induced by permanent occlusion of the distal part of the left MCA as previously described (Møller et al., 1995; Gregersen et al., 2000). Mice were anesthetized by s.c. injection of a mixture of Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml; Jansen-Cilag), Stesolid (5 mg/ml Diazepamum; Dumex) and distilled water (1:1:2; 0.20 ml/10 g body weight). Before surgery, the mouse was placed on a 37 °C±0.5 °C warm heating pad. Surgically, a skin incision was made from the eye to the ear of the mouse. The parotid gland and the upper part of the temporal muscle were pushed aside and a small hole was drilled over the distal part of the MCA. The MCA was occluded by electro-coagulation and the open incision was stitched with a 4.0 nylon-suture. In sham-operated mice the dura was opened over the MCA and the coagulator was applied to the brain parenchyma next to the MCA, but the artery was not occluded. After surgery, the mice were injected with 1 ml of isotonic saline and their eyes were coated with ointment. The mice recovered from the surgery in a recovery room at 28 °C. For treatment of post-surgical pain, mice were supplied s.c. with 0.15 ml Temgesic diluted 1:30 (stock: 0.3 mg/ml Buprenorphinum; Reckitt & Colman, UK) three times with an 8 h interval starting immediately after surgery.

Brain tissue processing

The mice used for *in situ* hybridization or RT rt-PCR were killed by cervical dislocation and the brains were carefully removed. The brains used for *in situ* hybridization were frozen in gaseous CO_2 and stored at −80 °C until serially sectioned into 30 μm thick coronal cryostat sections. Sections were placed on RNAse free object slides and stored at −80 °C until further use. The brains used for RT rt-PCR were removed from the skull, remaining meninges were removed, and the brain stem was transected at the level of the superior colliculi, after which the cerebral hemispheres and the remaining part of the brain stem were dissected into ipsilateral and contralateral halves. Each hemisphere was placed in 10 volumes of RNAlater RNA Stabilization Reagent (Qiagen, Germany).

Mice used for immunohistochemistry for IL-1β and double immunohistochemistry for IL-1β and macrophage associated antigen-1 (Mac-1/CD11b) were deeply anesthetized and perfused through the left ventricle, using 5 ml chilled 0.15 M Sørensen buffer (Sø-PB), pH 7.4, followed by 20 ml chilled 4% paraformaldehyde (PFA) in 0.15 M Sø-PB, pH 7.4. The brains were carefully removed and post-fixated in 4% PFA for 1 h, immersed in 20% sucrose overnight, frozen using gaseous CO_2 and stored at −40 °C until serially sectioned into 16 μm thick cryostat sections.

Determination of infarct volume

One series of brain sections from each SJL mouse was stained with Toluidine Blue, dehydrated in graded series of alcohol (96%–99% ethanol), cleared in xylene and coverslipped in Depex (BDH Gurr, UK). The infarct volume was estimated by counting all points hitting the infarct using the Computer Assisted Sterological Test (CAST) GRID microscope-system (Olympus, Denmark) and the Cavalieri principle for volume estimation (Møller et al., 1995; Gregersen et al., 2000). The total volume of the infarct (V_{total}) was calculated using the formula: $V_{total}=\Sigma P\times t\times a_{point}$, where ΣP is the total number of points hitting the infarct, t is the mean distance between sections, and a_{point} represents the area per point (Møller et al., 1995).

In situ hybridization for IL-1β mRNA-expressing cells

In situ hybridization for IL-1β mRNA-expressing cells was performed as previously described (Lambertsen et al., 2001). Frozen brain sections were dried for 10 min, at 55 °C and placed in 96% ethanol overnight, at 4 °C. The following day, sections were air dried and hybridized at 37 °C overnight in a hybridization buffer (50% formamide, 0.1 M Tris–HCl, pH 7.5, 4× saline sodium citrate [SSC; 1× SSC: 150 mM NaCl and 15 mM sodium citrate], 0.04 g polyvinylpyrrolidone Sigma-Aldrich, Denmark], 0.04 g bovine serum albumin [Sigma-Aldrich], 10% dextran sulfate [Pharmacia Biotech, Denmark], and single stranded salmon sperm DNA [Sigma-Aldrich]) containing an alkaline phosphatase (AP)-labeled oligonucleotide probe (10 pmol/ml IL-1β or 2 pmol/ml glyceraldehyde-3-phosphate dehydrogenase [GAPDH; DNA Technology A/S, Denmark]; Table 1). Post-hybridization, sections were rinsed in 1× SSC (pH 8.5) for 3×30 min, at 55 °C, followed by 2×10 min in