European Journal of Pharmacology 796 (2017) 131–138

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Methotrexate prevents epidural fibrosis through endoplasmic reticulum stress signalling pathway

Hui Chen[1], Lianqi Yan[1], Jingcheng Wang*, Yu Sun*, Xiaolei Li, Shuai Zhao, Daxin Wang, Gengyao Zhu, Yuan Liang

Department of Orthopedics, Clinical medical college of Yangzhou University, Subei People's Hospital of Jiangsu Province, Yangzhou 225001, China

ARTICLE INFO

Keywords:
Methotrexate
Endoplasmic reticulum stress
Fibroblast apoptosis
Epidural fibrosis

ABSTRACT

Lumbar laminectomy is one of the most common treatments for lumbar disc herniation and other lumbar disorders with serious complications, such as failed back surgery syndrome, mainly caused by epidural fibrosis (EF). The developing fibrosis causes radicular pain after the laminectomy or discectomy. Methotrexate (MTX) is a folic acid antagonist that has shown anti-proliferative effects in previous studies. The aim of our experiment is to study whether MTX has positive effects on the outcome of the laminectomy in rats. Our finding first demonstrated the beneficial effect of topical application of MTX in laminectomy models. As the results of a macroscopic scoring system, hydroxyproline content analysis, histological evaluation, the number of fibroblasts and immunohistochemistry showed that MTX suppressed the EF compared with the control group, and the inhibiting effect was in a dose-dependent manner. Furthermore, we hypothesized that the endoplasmic reticulum (ER) stress mediated the suppression effect of the EF. To verify this point of view, fibroblast cells cultured from epidural scar tissues of rats were used. CCK-8 assay, Western blot (for apoptotic genes, such as cleaved PARP) and annexin V-FITC/PI double-labelling showed that MTX could induce cell apoptosis. The expression of CHOP and GRP78 and the activation of ER stress-associated genes strongly suggested that ER stress mediated the apoptotic signalling pathway; immunohistochemistry of GRP78 and CHOP further verified this. Our findings indicate that topical application of MTX could indeed reduce EF, and the application of MTX could induce apoptosis through ER stress in rats.

1. Introduction

Lumbar laminectomy is one of the most common treatments for lumbar disc herniation and other lumbar disorders; however, it may cause a series of symptoms, including “failed back surgery syndrome” (FBSS) (Guyer et al., 2006; Emmez et al., 2008), which could result in poor clinical outcomes. These clinical outcomes include recurrent, persistent low back pain and disability (Xu et al., 2012). There are many etiologic reasons for FBSS, but epidural fibrosis (EF) after laminectomy is implicated as the main contributing factor (Siqueira et al., 1983; Ross et al., 1996). The developing fibrosis causes radicular pain by leading compression or tethering the nerve roots and impeding their normal motion, then these constrained nerve roots were stretched by the movement of the vertebral column (Andrychowski et al., 2013; Yang et al., 2011). Thus, preventing EF formation is believed to be the best approach to manage this problem. A number of methods have been studied to prevent EF, including surgical methods and material agents (Abitbol et al., 1994); however, effects of these treatments are not satisfactory, so their clinical applications are still limited. Therefore, preventing the occurrence of EF is still a great challenge to surgeons.

Many drugs, including some anti-cancer agents, such as mitomycin C (MMC), 5-fluorouracil (5-FU) and hydroxycamptothecin (HCPT) (Lee et al. 2004; Yildiz et al., 2007; Sun et al., 2008), and some immunosuppressive agents, such as tacrolimus (FK506) and pimecrolimus (Cemil et al., 2009, Yan et al., 2013), have been studied to prevent this condition, but experiments showed that they still have quite a lot of limitations before clinical trials. Methotrexate (MTX) is a folic acid antagonist (Belinsky et al., 2007), which is used in the treatment of cancer chemotherapy, rheumatoid arthritis and psoriasis (Arena et al., 2012a). Recently, it was reported that MTX induces apoptosis in CCRF-CEM and Nalm6 cells through the activation of endoplasmic reticulum (ER) stress (Kuznetsov et al., 2011).

The ER is the place where protein synthesis, folding, assembly and

* Corresponding authors.
E-mail addresses: jingchengwyz@163.com (J. Wang), docyusun@126.com (Y. Sun).
[1] Hui Chen and Lianqi Yan equally contributed to paper.

http://dx.doi.org/10.1016/j.ejphar.2016.12.032
Received 14 November 2016; Received in revised form 12 December 2016; Accepted 20 December 2016
Available online 21 December 2016

transport occurs (Marciniak and Ron, 2006). When the unfolded/misfolded proteins assemble under a certain degree, the unfolded protein response (UPR) provides a protective effect to the cells from the injury and then restore regular functions (Lenna and Trojanowska, 2012). However, chronic stress or a failed adaptive response in the ER causes apoptosis by triggering the accumulation of glucose-regulated protein 78 (GRP78/BiP) and activation of the dsRNA-activated protein kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and transcription factor 6 (ATF6). Then the PERK phosphorylates eukaryotic initiation factor-2 (eIF2α), regulates the expression of ER stress target genes, including CCAAT/enhancer binding protein homologous protein (CHOP) (Gotoh et al., 2004; Tajiri et al., 2004). The Bcl-2 family is the downstream of CHOP, which plays a vital role in the ER stress-mediated cell death pathways (Hetz, 2007).

Therefore, we are interested in whether MTX induces fibroblast apoptosis by ER stress and whether it could reduce and prevent EF, in the hopes of treating EF in the future.

2. Materials and methods

2.1. Fibroblast culture and treatment

Fibroblast cells were obtained from epidural scar tissue isolated from rats that underwent laminectomies. Fibroblasts were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY), supplemented with 20% foetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (PS; Thermo, Rockford, IL). Cells in exponential growth phase between passages 3 and 6 were used for the experiments. Fibroblast cell monolayers were seeded into 96-well plates, 6-well plates, or 10-cm dishes overnight until reaching approximately 50–80% density and then were washed with phosphate-buffered saline (PBS; pH 7.4) and treated with MTX purchased from Sigma (St. Louis, MO, USA) in various concentrations of reagent and in various times. The controls were treated with 5-min applications of PBS only. After treatment, the cells were immediately washed three times with PBS and were maintained in the growth medium for subsequent experiments.

2.2. Cell viability

Cell viability was measured using a Cell Counting Kit-8 (CCK-8;Dojindo, Tokyo, Japan). The cells were cultured in triplicate in 96-well plates and were treated with 10^{-5}–10^{-9} M MTX. In another group, the cells were maintained in DMEM for 0, 12, 24, 36, 48 or 72 h, as described previously, and the cells were further incubated with 10 μl WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfonyl]-2H-tetrazolium; Dojindo Laboratories, Kumamoto, Japan) for 1 h at 37 °C. Cells that stained positively with WST-8 were considered viable cells and were expressed as a percentage compared with the control cells.

2.3. Western blot analysis

Treated cells were lysed on ice in lysis buffer (Beyotime, Hangzhou, China), according to the manufacturer's instructions. The protein concentration was determined by the BCA Protein Assay Kit (Beyotime, Hangzhou, China). Equal amounts (60 μg/lane) of total proteins were subjected to electrophoresis on a 6%, 10% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk in tris-buffered saline and Tween 20 (TBST) for 2 h at room temperature and then incubated with the primary antibodies. Anti-78-kDa glucose-regulated protein (GRP78), anti-CHOP, anti-phosphoIRE1α (S724) antibodies, anti-activating transcription factor 6 (ATF6), anti- phospho-eIF2α, anti- eIF2α, anti-Bax and anti-Bcl-2 antibodies were obtained from Abcam (Abcam, Hong Kong, China). Anti-phospho-PERK and anti-inositol-requiring enzyme 1α (IRE1α) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-poly ADP-ribose polymerase (PARP), anti-PERK and anti-β-actin antibody were purchased from Cell Signalling Technology (Cell Signalling Technology, Beverly, MA, USA). The membranes were next washed three times in TBST and incubated with horseradish peroxidase-conjugated goat antimouse or anti-rabbit IgG (Santa Cruz Biotechnology) (diluted 1:5000) for 2 h and then washed with TBST three times. The immune complexes were visualised via fluorography enhanced by the electrochemiluminescence system (Millipore, Bedford, USA).

2.4. Flow cytometry analysis of cell apoptosis

The cells were plated in 6-well plates and incubated overnight at 37 °C. After treatment with 1 μM MTX for 24 h, the detached and adherent cells were collected and washed three times with ice-cold PBS buffer. Cells were then resuspended in binding buffer at 1×10^6 cells/well and incubated with annexin V-FITC and PI (BD Biosciences, Singapore) for double-staining, according to the manufacturer's protocol. Before the analysis, the mixture was incubated in the dark for 15 min at room temperature.

2.5. Animals

A total of 48 Sprague-Dawley, young-adult, male rats (purchased from the experimental animal centre of Yangzhou university, China) with weights of 250 ± 20 g were used for this study. All animals received care in compliance with the principles of Laboratory Animal Care of international recommendations, and the experimental protocol was approved by the Animal Care and Research Committee of the Yangzhou University, China. The rats were randomly divided into four groups (12 rats per group): MTX (0.5 mg/ml), MTX (1 mg/ml), MTX (2 mg/ml) or control (saline). The rats were acclimated to the environment for 1 week before the experiment.

2.6. Animal mode

Laminectomy models were performed on rats according to the procedure in the previous study (Sun et al., 2007). After anaesthesia by intraperitoneal injection of ketamine (100 mg/kg body weight), the fur around the location of L1 and L2 were shaved, then antisepsis of the exposed skin was performed with iodophor. The L1 vertebral plate was exposed by a midline skin incision and separation of the paraspinal muscles. The dura mater was exposed after removing the spinous process and vertebral plate of L1 by rongeur forceps. A total laminectomy of L1 was performed.

2.7. Topical application of drugs

After depilation, disinfection and hemostasis of the lumbar region, MTX in various concentrations of 0.5, 1 and 2 mg/ml or saline were administered to the laminectomy areas with cotton pads (4×4 mm) for 5 min. The surrounding tissues were covered by wet gauzes to avoid touching the agent. After the cotton pads were removed, the decorticated areas of laminectomy were irrigated with saline to get rid of the remaining MTX immediately. The wounds were then sutured to close. After the operations, an intramuscular injection of Cefazolin sodium was administered for 3 days to the rats to prevent infection after the operation.

2.8. Macroscopic assessment of EF

Six rats were randomly selected from each group after 4 weeks for macroscopic evaluation. The surgical sites were reopened, and epidural