Osteopontin alters the functional profile of porcine microglia in vitro

Bart R. Tambuyzer¹, Christophe Casteleyn, Hans Vergauwen, Steven Van Cruchten and Chris Van Ginneken
Laboratory of Applied Veterinary Morphology, Department of Veterinary Sciences, University of Antwerp, Wilrijk, Antwerp, Belgium

Abstract

OPN (osteopontin) is a secreted glycoprotein predominantly expressed in bone matrix and kidney tissue. More recently, a neuroprotective role has been attributed to this cytokine since it can be up-regulated by microglia in neurodegeneration and inflammation. We demonstrate the expression of OPN within primary cultured microglia. Microglia incubated in vitro with different concentrations (0.1 fM–1 nM) of recombinant OPN showed increased proliferation at 10 fM. Moreover, conditioned medium of LLC-PK1 cells, a pig renal epithelial cell line and a known source of secreted OPN, more than doubled the rate of proliferation of microglia. Addition of an anti-OPN polyclonal antibody completely reversed this effect. Treatment with OPN dose-dependently also inhibited microglial superoxide production. In contrast, phagocytosis of fluorescent-labelled beads was enhanced by OPN. In conclusion, OPN shifts microglia, at least in vitro, to an alternative functional profile more fit to the immune-balanced microenvironment of the CNS (central nervous system).

Keywords: microglia; osteopontin; phagocytosis; proliferation; superoxide

1. Introduction

Microglia are dispersed as ramified resting cells throughout the CNS (central nervous system) and fulfill an immune sentinel function as they constantly scan their microenvironment. During inflammation microglia transform into an activated phenotype equipped with a broad spectrum of immune effector functions. They become phagocytic, have the potential to proliferate and secrete cytokines, chemokines, ROS (reactive oxygen species), proteases, etc. This allows them to move through CNS tissue, target and eliminate apoptotic/necrotic cells or pathogens, and communicate with neurons and other glia in the immune-balanced CNS microenvironment (Tambuyzer et al., 2009).

OPN (osteopontin) is a secreted glycoprotein mainly produced in bone and kidney tissues (Giachelli and Steitz, 2000), but can also be expressed by inflammatory cells, including macrophages. Although the expression of OPN is low under physiological conditions, it is readily up-regulated during CNS inflammation (Ellison et al., 1999; Hashimoto et al., 2007) or neurodegeneration (Maetzler et al., 2007; Comi et al., 2010). However, OPN expression is not solely pathology-associated as ameboid microglia transiently express OPN during the development of the rat brain (Choi et al., 2004). Additionally, for macrophages that are functional and ontological related to microglia (Tambuyzer et al., 2009), OPN has been implicated in adhesion, migration, phagocytosis, differentiation as well as in the production of oxygen radicals and cytokines (Giachelli and Steitz, 2000). However, the effect of OPN on microglia, as an autocrine and paracrine factor, remains unresolved. In this study, we have investigated the in vitro effects of recombinant OPN on the functional profile of porcine neonatal microglia.

2. Materials and methods

2.1. Characterization of microglia and OPN immunofluorescence

Primary microglial cells were prepared from mixed cortical glial cell cultures as previously described (Tambuyzer and Nouwen, 2005). All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp. For fluorescence microscopy, microglial cells adhered onto Permanox™ slides (Nunc) were fixed [4% PFA (paraformaldehyde) in PBS, 10 min] and labelled with a biotinylated lectin RCA-I (Ricinus communis agglutinin-I; 5 µg/ml, Vector Laboratories) and a polyclonal rabbit anti-human OPN antibody (1:10, Abcam) followed by labelling with streptavidin-AlexaFluor568 (1:200, Invitrogen) and a FITC-conjugated goat anti-rabbit Ig antibody (1:200, Jackson Immunchemicals). Images were taken with a fluorescent microscope (Olympus) equipped with a digital camera (DP71, Olympus), and analysed using cellSense™ image analysis software (Olympus). For flow cytometric analysis, harvested microglia were labelled with biotinylated RCA-I (5 µg/ml), anti-porcine CD172a/SWC3 (1:200), anti-porcine CD4 (1:10) or anti-porcine CD8a antibody (1:10) (all mouse antibodies from Serotec), followed by labelling with streptavidin-RPE (1:10, Sigma) or RPE-labelled goat anti-mouse Ig antibody (1:200, Jackson Immunchemicals) for the CD markers. Cell viability was assessed through addition of GelRed (1 x final concentration, Biotum) to the cell suspension immediately before flow cytometric analysis. Samples were analysed with an Epics XL-MCL analytical flow cytometer (Beckman Coulter) and the data were processed using the WinMDI software package (Joseph Trotter/Scripps FACS software).

¹ To whom correspondence should be addressed (email bart.tambuyzer@ua.ac.be).

Abbreviations: CNS, central nervous system; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; OPN, osteopontin; RCA-I, Ricinus communis agglutinin-I; ROS, reactive oxygen species.
2.2. Proliferation assay

In vitro proliferation of microglia was monitored using $^3$H-thymidine incorporation. Microglia were incubated in a 96-well microtiter plate ($2.5 \times 10^4$ cells/well). The cells were incubated with recombinant human OPN (R&D systems), diluted to different concentrations (0.1 fM–1 nM) in DMEM (Dulbecco’s modified Eagle medium) complete medium with 10% FCS (fetal calf serum), Penicillin/Streptomycin (100 units/ml and 100 µg/ml, respectively), Gentamycin (100 µg/ml) and Fungizone (0.25 µg/ml) (Invitrogen) during a 4-day period. Alternatively, microglia were incubated with LLC-PK1 (porcine proximal tubular epithelial cell line, CL-101, ATTC, LGC Promochem)-conditioned medium (CM-PK1: Tambuyzer et al., 2007) to which a polyclonal anti-human OPN antiserum was added at different concentrations (1:100–1:1000; Abcam). For this experiment CM-PK1 served as positive control condition (c+) and DMEM complete medium as a negative control (c-). $^3$H-thymidine (80 µCi/ml) (GE Healthcare) was added to the wells after 48 h. DNA from microglia was harvested after 96 h with a Skatron Cell Harvester (MDC) and transferred onto a glass fibre filter mat (MDC). $^3$H-thymidine radioactivity on the filters was counted by means of a liquid scintillation cocktail (EcoLite+, ICN) with a Liquid Scintillation Counter (Packard) as DPM (disintegrates per minute) for each filter (= well). Data are expressed as mean relative % incorporation ± S.E.M. with control (c-) at 100%.

2.3. Superoxide production assay

Microglia were harvested from mixed glial cultures and plated in 96-well plates ($2.5 \times 10^4$ cells/well). The cells were incubated 4 days in DMEM (= controls) or recombinant human OPN at different concentrations (10 fM–1 nM) in the DMEM complete medium with or without FCS supplementation. Microglial superoxide production upon PMA-induction (500 nM, Sigma) was quantified by the SOD (superoxide dismutase)-inhibitable reduction of ferricytochrome c with a specific absorption at 550 nm (Tambuyzer and Nouwen, 2005).
2.4. Phagocytosis assay

Microglia (2 × 10^5 cells/well) were incubated in the control medium (DMEM complete medium) or treated with OPN at different concentrations (0.1 pM–1 nM) in DMEM for 24 h in a 96-well plate coated with semi-solid agar as described earlier (Tambuyzer et al., 2007). Phagocytosis was assessed by ingestion of FITC-labelled fluorescent beads (1 μm, Polysciences) (bead/cell ratio 100:1) over 1 h at 37°C in air plus 5% CO₂. Uptake of beads was confirmed earlier with confocal laser-scanning microscopy (Tambuyzer et al., 2007) and could be inhibited with 5 μM cytochalasin D (Sigma). The cells were washed to remove non-adherent beads, resuspended in PBS (0.2% NaN₃, 0.2% EDTA) and analysed using an FACSCAN flow cytometer (BD). Data were processed using WinMDI software.

2.5. Statistics

Data were analysed statistically by the Kruskal–Wallis test and with the Mann–Whitney U-test for post hoc comparison using SPSS. P<0.05 was considered statistically significant. Data are presented from at least three independent experiments.

3. Results and discussion

3.1. Identification of microglia and OPN immunostaining

The purity of the microglia cultures, derived from the mixed cerebral cortical cultures, as determined by RCA-I lectin staining was >95% (Figure 1). Moreover, microglia harvested from mixed cultures were CD4−CD8−, and CD172α+/SWC3+ (Figure 1), corroborating an earlier report on porcine retinal microglia (Yang et al., 2002). These cells have previously been shown to be CD11b+CD45+, produce acid phosphatase, non-specific esterase and nucleotide diphosphatase, and bind the lectins RCA-I and GS-Ib4 (Tambuyzer and Nouwen, 2005; Tambuyzer et al., 2007; 2012). Almost all microglial cells from the monocultures positively immunostained for OPN, which was verified with RCA-I/OPN double staining (Figure 2). The data demonstrate that in vitro cultured microglia can also produce OPN, corroborating earlier reports on microglial OPN expression in hippocampal slice cultures (Lee et al., 2010), as on its in vivo expression during the CNS development (Choi et al., 2004) and neuropathology (Ellison et al., 1999; Maetzler et al., 2007). All cultures showed high viability (>95%) at all time-points or conditions as monitored by Trypan Blue exclusion or GelRed staining.

3.2. OPN stimulates proliferation of microglia

Microglia showed increased proliferation compared with control cells (P<0.005) when a low concentration (10 fM) recombinant OPN was added to the culture medium (Figure 3A). An augmented proliferative activity had already been demonstrated for epithelial cells after OPN treatment (Elgavish et al., 1998). Moreover, OPN has been associated with tumour proliferation (Hsu et al., 2010). Conditioned medium from LLC-PK1 cells (CM-PK1, c+) increased 3H-thymidine incorporation by microglial cells by almost 2.5 times compared with the control medium (c−, Figure 3B), an effect described earlier (Tambuyzer et al., 2007). Moreover, this effect was markedly higher than with recombinant human OPN. As human and porcine OPN are only 69% identical and show 79% sequence similarity, porcine OPN in CM-PK1 might induce a stronger response of porcine microglia. This proliferation promoting effect was completely and dose-dependently blocked by a polyclonal anti-OPN antiserum added to the conditioned medium (Figure 3B). We hypothesize that the proliferation promoting activity of CM-PK1 is at least in part due to its OPN content. Indeed, LLC-PK1 cells can express and secrete OPN (Kahn and Thamilselvan, 2000). Although OPN can exert its effects at low concentrations, relatively high concentrations of anti-OPN antibody were needed to inhibit the effects of CM-PK1 medium. However, the OPN content of CM-PK1 is unknown, whereas it might be possible that the amount of OPN produced by LLC-PK1 cells exceeds the quantity of anti-OPN antibody capable of blocking the proliferation stimulating effect. Moreover, the antibody used was polyclonal, and the proportion of this antibody acting specifically on the receptor interacting-epitope, remains
elusive. In this way, dilution can have differential effects on its inhibitory properties.

### 3.3. OPN inhibits microglial superoxide production

Microglia cells treated for 4 days with recombinant OPN (10 fM–1 nM) and subsequently stimulated with PMA showed a significant inhibition of superoxide production (Figures 4A and 4B). This occurred at lower OPN concentrations (10 fM) when the microglia cells were grown in the absence of foetal bovine serum (Figure 4A). With serum present, microglial superoxide production was significantly inhibited only at a higher OPN concentration (10 pM; Figure 4B). These experiments could mimic the normal CNS environment (serum-free) and its disturbance during neuro-pathology with blood–brain barrier disruption. Accordingly, OPN may have neuroprotective properties during stroke (Ellison et al., 1999; Meller et al., 2005).

#### 3.4. OPN enhances phagocytosis by microglia

Freshly harvested microglia initially had a high phagocytic activity, on which OPN treatment had no significant effect (Figure 5). After 24 h culture in the DMEM complete medium, their phagocytic activity was reduced to 40% of this initial level. However, when these cells were incubated for 24 h with OPN, a significant increase in phagocytotic activity occurred compared with microglia cultured in control medium. The uptake of beads by microglia treated with 1 nM OPN was almost doubled that of control cells, thereby largely restoring the activity to the level observed immediately after harvesting. OPN has been correlated with increased phagocytosis by brain macrophages in a rat stroke model (Shin et al., 2011), and also by peripheral monocytes/macrophages, whereas this protein can act as an opsonin (Schack et al., 2009).

The data are analogous to findings in our recent report on IFN-γ (interferon-γ), where a neuroprotective potential was attributed to a pro-inflammatory cytokine (Tambuyzer et al., 2012). Indeed, OPN has also been designated a pro-inflammatory (neurotoxic) cytokine (Chabas et al., 2001). In contrast, absence of OPN leads
to less inflammation, but more tissue damage, in spinal cord injury (Hashimoto et al., 2007). To this extent, it has been shown that OPN is a promoter of inflammation (by recruitment of macrophages), but an inhibitor of oxidative stress (Wolak et al., 2009). Wolak and colleagues indicate that deletion of OPN augments NADPH-oxidase protein expression and consequently increases oxidative stress. OPN itself has been designated an 'oxidant stress sensitive cytokine' (Urtasun et al., 2012) that can be up-regulated by oxidative stress (Mazière et al., 2010). Therefore it is conceivable that OPN, as an oxidative stress-regulated protein, provides a negative feedback for oxidative metabolism in inflammatory cells or could even directly scavenge oxygen radicals.

In conclusion, OPN is expressed by porcine microglia in vitro. Our data support a dual role, since proliferation and phagocytosis of microglia could be increased, but production of neurotoxic ROS is inhibited, leading to a phenotype more fit to the immune-balanced CNS environment, causing less bystander damage. These findings may lead to a better understanding of the various autocrine and paracrine influences of OPN on microglia in neuropathology and development of the CNS.

Author contribution

Bart Tambuyzer drafted and revised the paper, performed cell function experiments, acquisition, processing and interpretation of data on cell function assays. Christophe Casteleyn performed immunofluorescence experiments, acquisition and processing of images, and revised the paper. Hans Vergauwen performed FACs experiments, analysis and interpretation, and revised the paper. Steven Van Cruchten performed statistical processing and interpretation of data, and revised the paper. Chris Van Ginneken provided the conceptual design of the study and interpreted the data, and drafted and revised the paper. All authors approved the final version of the paper.

Acknowledgements

We thank Professor P. Ponsaerts for the use of the flow cytometer and fluorescence microscope at the Laboratory of Experimental Hematology.

Funding

This work was funded by the Belgian Fund for Scientific Research (FWO, grant No. G.0444.03).

References


Hashimoto M, Sun D, Ritting SR, Denhardt DT, Young W. Osteopontin-deficient mice exhibit less inflammation, greater tissue damage, and impaired locomotor recovery from spinal cord injury compared with wild-type controls. J Neurosci 2007;27:3603–11.


Received 20 March 2012/17 July 2012; accepted 13 September 2012

Published as Immediate Publication 13 September 2012, doi 10.1042/CBI20120172