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Research Report

Proinflammatory cytokine production by cultured neonatal rat microglia after exposure to blood products

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ABSTRACT

Periventricular germinal matrix hemorrhage is a devastating complication of preterm birth. Inflammation appears to play a role in brain damage after premature birth and hypoxia. The effects of rat blood plasma and serum on cytokine expression by cultured rat microglial cells were investigated. We analyzed mRNA expression levels of tumor necrosis factor (TNF)-α, interleukin-6 and protease activated receptor-1 and -4 by quantitative RT-PCR. Protein expression for TNFα was done using immunocytochemistry and ELISPOT assays. Plasma and serum had dose dependent toxic effects on microglia as measured by lactate dehydrogenase release assay and activated caspase-3 immunocytochemistry. High concentrations of plasma enhanced TNFα mRNA expression and protein production, while high concentrations of serum enhanced IL-6 mRNA expression. This study suggests that soluble components of blood might be differentially responsible for up regulating production of the cytokines TNFα and IL-6 by microglia from immature rodent brain.

1. Introduction

Preterm infants are at risk for periventricular hemorrhage (PVH) in the brain. Hemorrhage in the mature brain causes damage through direct physical disruption, proteolytic activity of enzymes involved in blood clot formation (e.g. thrombin and plasmin) (Xue and Del Bigio, 2001; Xue and Del Bigio, 2005a,b), hemoglobin released from lysed erythrocytes (Xi et al., 1998), and inflammation and cytokine production (Xi et al., 2006; Xue et al., 2003a,b). We have shown in neonatal mouse brain that an inflammatory response including microglial activation occurs soon after blood injection into the periventricular region (Xue et al., 2003a,b). In the same model, activation of the systemic immune system prior to blood injection aggravates the brain injury (Xue et al., 2005). We have also shown that injection of thrombin or plasmin into the periventricular region of mouse brains is associated with dose dependent tissue necrosis, cell death and inflammation at 48 h (Xue et al., 2005).

Microglia, which are derived from cells of the monocytic lineage, play a critical role in immune reactions in the central nervous system (CNS), in part through their ability to secrete inflammatory mediators (Farber and Kettenmann, 2005). Activated microglia in damaged white matter (periventricular leukomalacia) of infants born prematurely exhibit strong immunostaining for tumor necrosis factor alpha (TNFα) and other cytokines (Folkerth et al., 2004; Kadhim et al., 2001). TNFα has

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Abbreviations: IL6, interleukin 6; LDH, lactate dehydrogenase; mRNA, messenger ribonucleic acid; PAR, protease activated receptor; PVH, periventricular hemorrhage; qRT-PCR, quantitative (real time) reverse transcriptase polymerase chain reaction; TNFα, tumor necrosis factor alpha

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pleotropic effects on glia and neurons. It regulates the continued production of TNFα, interleukin-6 (IL-6), and interleukin 1 beta (IL-1β) through an autocrine feedback system (Kuno et al., 2005). Enhanced IL-6 production related to genetic variations in the promoter region is associated with worse brain damage after preterm birth (Harding et al., 2004). Furthermore, the IL-6 receptor is highly expressed in the ganglionic eminence (Ulfig and Friese, 1999), which is the major site of periventricular hemorrhage. Theoretically, proteins from blood can diffuse from sites of hemorrhage into surrounding white matter where they might aggravate hypoxic damage.

We hypothesized that soluble blood components would induce cytokine production by microglia isolated from newborn rat brain. This developmental stage is roughly comparable to that of premature humans at risk for periventricular hemorrhage (Bayer et al., 1995). Our in vivo experiments showed that thrombin and plasmin can damage the neonatal mouse brain to a greater extent than whole blood (theoretically) containing the same amount of these proteolytic enzymes (Xue et al., 2005). We speculated that inhibitors such as antithrombin, alpha2-macroglobulin, and alpha1-antitrypsin might be important in the inactivation of plasma proteolytic proteins. We wanted to determine if plasma and serum have different effects on microglial activation. Plasma is the liquid component of blood with cellular elements removed, while serum is defined as the liquid remaining after blood has clotted. We expected that plasma might have effects on microglia because of the greater availability of proteolytic enzymes. We used quantitative (real time) reverse transcriptase PCR (qRT-PCR) to determine the effect of plasma and serum on TNFα and IL-6 mRNA expression by microglia. We then examined microglia to determine whether they are primed to produce TNFα when exposed to blood in vivo. In addition, we have examined the response of protease activated receptor (PAR) type 1 and type 4. PARs (also known as thrombin receptors) are widely expressed in the central nervous system. Thrombin activates PAR-4 by cleavage at Arg^{47}/Gly^{48} in the N-terminal sequence. However, PAR-4 activation requires considerably higher levels of thrombin than for blood clotting, possibly due to the lack of the hirudin-like thrombin binding sequence (Xu et al., 1998). Activation of PAR-1 contributes to microglial activation, brain inflammation and neuronal damage (Junge et al., 2003; Suo et al., 2002).

2. Results

Isolated rat microglial cells were 95% pure as indicated by CD45 immunolabeling on the cell membranes (Fig. 1). Microglial cells grown in the control culture medium were fusiform with rare delicate processes (Fig. 2). Addition of rat plasma or serum at high concentrations (1:1 dilution) to the culture medium resulted in extensive cell death as indicated by separation of most cells from the plate by 24 h. Addition of plasma or serum in 1:10–1:100,000 dilutions caused the cells to convert to ameboid morphology with flattened cell bodies by 24 h. Nuclear clumping was suggestive of an apoptotic mode of cell death (Fig. 2). LDH activity was significantly higher in the supernatant of cells treated with 1:1 dilution of plasma and serum (Fig. 3). Activated caspase-3 immunocytochemistry also showed that plasma and serum induce cell death in a dose dependent manner (Fig. 3). The extent of cell death was significantly greater in cells treated with plasma compared to serum.

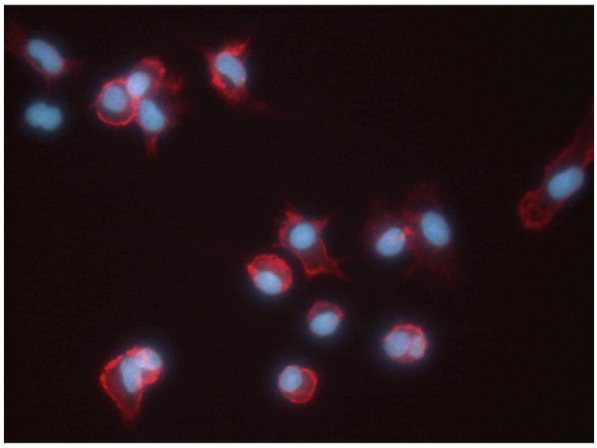

Fig. 1 – Photomicrograph showing cultured rat microglial cells immunolabeled for CD45 (Cy3 red fluorescence localized to membranes), a marker of microglial/ leukocyte lineage. DAPI (blue fluorescence) was used for nuclear counterstaining. The culture is 95% microglia. (400× magnification).

Quantitative RT-PCR showed that TNFα mRNA expression was very low in control cells. High concentrations of serum did not affect TNFα expression significantly (Fig. 4). However, microglia grown in medium that contained plasma in 1:1 and 1:10 dilutions showed a significant increase in TNFα mRNA expression. IL-6 mRNA expression was barely detectable in control cells. IL-6 mRNA expression was significantly enhanced in cells grown in medium containing 1:1 serum (Fig. 4), but not in higher dilutions. Plasma had no effect on IL-6 mRNA expression at any concentration used. Serum at 1:1 and 1:10 dilutions significantly enhanced PAR-1 mRNA expression, whereas plasma did not alter PAR-1 mRNA expression compared to control (Fig. 5). PAR-4 mRNA expression was significantly higher in cells treated with high concentration of plasma compared to control. We also observed a dose dependent effect of plasma on PAR-4 mRNA expression (Fig. 5).

Immunocytochemistry confirmed the presence of TNF-α protein in the cells. Immunoreactivity was confined to cytoplasmic organelles (Fig. 6), possibly Golgi and endoplasmic reticulum (Beil et al., 1995; Shurety et al., 2000). Cells stimulated with plasma at 1:1 to 1:100 dilution had enhanced TNF-α protein expression compared to the controls, while serum had no effect.

Using the ELISPOT assay, we observed that microglia secreted detectable amounts of TNFα only if stimulated with lipopolysaccharide (LPS) in the culture medium. In comparison to microglia isolated from intact newborn rat brains, there was an increase in the number of TNFα secreting cells isolated from brains that had been subjected to blood injection 8 h prior to isolation (Fig. 7).