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BRAIN RESEARCH

Research Report

Hemoglobin crystals: A pro-inflammatory potential confounder of rat experimental intracerebral hemorrhage

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ABSTRACT

In vivo rat hemoglobin crystallization has been reported in lung, liver and kidney, but never following central nervous system injury. In the present study, we examined hemoglobin crystallization following experimental intracerebral hemorrhage (ICH) and its effects on inflammation. Ninety-one rat brains, subjected to either autologous or collagenase ICH, and vehicle controls, were retrospectively examined. In both models, hemoglobin crystals were present in most brains at 24 and 48 h. They were especially prominent at 24 h in autologous ICH brains (2.5% of the hematoma vs 0.6% in collagenase animals; $p=0.0001$) and, at 5 h, were only present in autologous ICH brains. Crystals were diminishing at 48 h and were absent at 7 days. Crystals appeared in clusters around blood vessels. In both models, at 24 h, crystals appeared strongly chemotactic for neutrophils. This effect was most pronounced in autologous ICH brains (2628 ± 182 neutrophils/mm^2 hematoma crystals vs 327 ± 54 neutrophils/mm^2 hematoma; $p<0.0001$). In these animals up to 30% of the total neutrophilic infiltrate was located around crystals. A greater overall neutrophilic infiltrate was seen in autologous ICHs with higher percentages of crystalline hemoglobin ($p=0.04$ for trend). Although hemoglobin crystallization occurs in both models of ICH, it is particularly prominent following autologous ICH. Accordingly, hemoglobin crystallization may exaggerate the importance of inflammation in this model.

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1. Introduction

Animal models of intracerebral hemorrhage (ICH) imperfectly mimic human disease (James et al., 2008). The most widely-used models are autologous blood (Whisnant et al., 1963) or collagenase injections (Rosenberg et al., 1990) into the brains of mice or rats (James et al., 2008). The pathological responses to these two models ICH differ significantly (MacLellan et al., 2008). Additionally, the pathophysiology of both of these models is known to differ in several ways from human ICH (for instance, the tempo of edema formation (Xi et al., 2006) and hematoma resorption (MacLellan et al., 2008)).

In this report we demonstrate a further difference, both between models and between rodent and human ICH: the presence of hemoglobin crystallization, which appears to evoke an exaggerated inflammatory response.

Whereas hemoglobin from humans and most other species crystallizes in vivo only if structurally abnormal, it has been known for some decades that rat hemoglobin readily crystallizes in vitro (Squires and McFadzean, 1963) and may even exist

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Abbreviations: ICH, Intracerebral hemorrhage; EM, Electron microscopy; H and E, Hematoxylin and eosin; DAB, Diaminobenzidene

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in a partially crystalline state within erythrocytes (Brunori et al., 1982). In vivo rat hemoglobin crystallization was first noted incidentally after toxin injury to lung (Harris and Chen, 1970), and characterized more completely following injury (graded pressure) to dentition (Rygh and Selvig, 1973). In this setting, hemoglobin crystals were noted after only 2 h of pressure; they became increasingly prominent by 24 h, but were absent at 60 h. The authors concluded that they represented an intermediate state of rat hemoglobin break-down. There have been several subsequent reports of hemoglobin crystallization following injury of varying etiologies to rat lung (Ghio et al., 2000; Paakko et al., 1996; Zachary et al., 2001) and kidney (Madsen et al., 1982), but never following central nervous system injury. In these studies hemoglobin crystals have been deemed a curiosity without pathophysiological significance. In the current study we show that hemoglobin crystallization occurs in rats following both autologous and collagenase ICH, and is particularly prominent following autologous ICH. Furthermore, we demonstrate that hemoglobin crystallization appears to exaggerate the ICH-induced inflammatory response.

2. Results

2.1. *Time-course and appearance of crystals in relation to hematoma*

At early timepoints H and E stained sections through the hematoma centre demonstrated crystalline structures (Figs. 1a, b). At 5, 24 and 48 h a greater percentage of the hematoma area was occupied by crystalline structures in the autologous ICH group than in the collagenase group (Table 1). In both models crystals were most prominent at the 24 hour timepoint (Table 1). Crystals were evident at 5 h in 4/5 autologous ICH animals (but not in 5 collagenase ICH animals). At 24 h they were present in 5/5 autologous ICH animals and 13/15 collagenase ICH animals and at 48 h in 4/5 autologous and 3/5 collagenase ICH animals. Crystals in both groups appeared to be disintegrating by 48 h (Figs. 1c, d) and no crystals were visible by 7 days in either group (Table 1), in rough parallel with hematoma resorption (Fig. 2a).

Crystals were tetragonal in shape and ranged from 0.5 to 20 μm in width and up to 166 μm in length. Crystals were commonly clustered around blood vessels (Fig. 1a), were eosinophilic, and, like rat erythrocytes, were sometimes pitted.

0.2 U of bacterial collagenase has previously been reported to cause a similar hematoma volume to 100 mcL autologous blood (MacLellan et al., 2008), however final autologous ICH hematoma volumes were around 30% smaller in our experiments (Fig. 2a). This did not appear to influence crystal formation, however, as, in the collagenase group, which has an inherent variability of hematoma size (as blood is not directly injected) there was no correlation between hematoma size and the percentage of the hematoma occupied by crystals (Fig. 2b).

2.2. *Identification of crystals*

Under light microscopy, microscopy eosinophilic staining of crystals appeared identical to adjacent erythrocytes. This characteristic, as well as a 'pitted' appearance, has previously

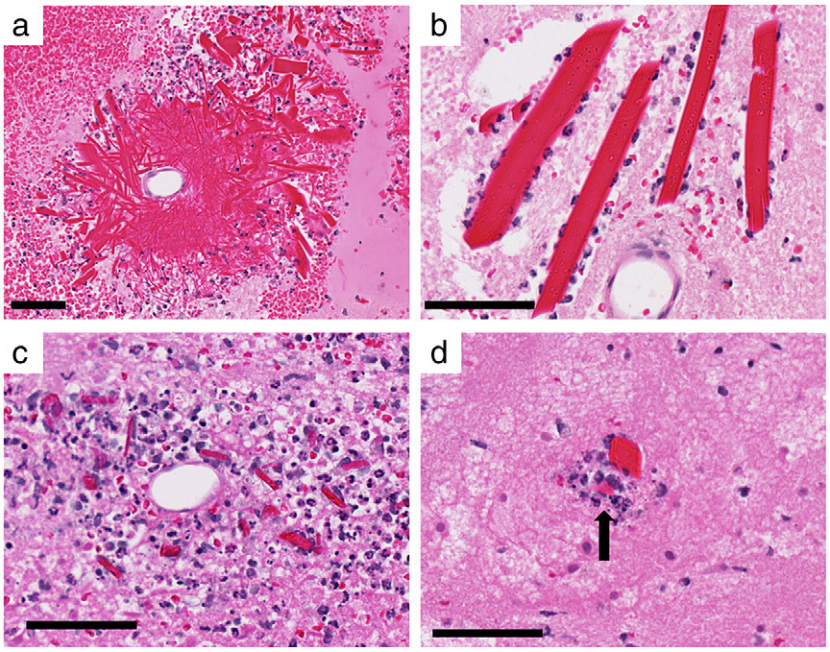

Fig. 1 – Staining of crystals following rat ICH. Scale bar 100 μm. (a) H and E, rat striatum, autologous ICH, 24 h. A large perivascular crystal cluster between areas of intact and lysed erythrocytes. Most visible neutrophils are closely associated with crystals; (b) H and E, rat striatum, collagenase ICH, 24 h. Large crystals amongst ghost erythrocytes, surrounded by palisading neutrophils; (c) H and E, rat striatum, autologous ICH, 48 h. A perivascular cluster of disintegrating crystals, surrounded by leucocytoclastic nuclear debris; (d) H and E, rat striatum, collagenase ICH, 48 h. A disintegrating crystal (arrow) adjacent to a better preserved crystal, both surrounded by leucocytes.

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