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α_1-Acid glycoprotein modulates apoptosis in bovine monocytes

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Abstract

α_1-Acid glycoprotein (AGP, orosomucoid) is a normal costituent of bovine blood. AGP is an immunocalin, a binding protein that can also exert several immunomodulatory functions. In this paper we investigated the effect of bovine α_1-acid glycoprotein (boAGP) on spontaneous and staurosporine-induced apoptosis of blood derived monocytes purified using magnetic cell sorting techniques. Bovine AGP was purifed from blood following a chromatographic protocol. The homogeneous protein was used to stimulate the cells as well to raise a polyclonal antibody, that was used throughout all the experiments. When monocytes were incubated with high concentrations of boAGP (0.9 mg/ml), similar to those found in bovine plasma during systemic reaction to inflammation, their spontaneous apoptosis rate was suppressed, as determined by caspase-3/7 enzymatic activity assay. Similar results were obtained when apoptosis was induced by adding staurosporine, a potent protein kinase inhibitor. The apoptosis-modulating activity of boAGP was dependent on its concentration, since physiological concentrations of boAGP (0.3 mg/ml) did not exhibit a statistically significative anti-apoptotic activity. We also investigated whether this apoptosis-modulating activity was dependent on the terminal sialic acid residues exposed on the surface of the protein. Enzymatic treatment with neuraminidase, that cleaves terminal sialic acid residues, completely abolished boAGP's anti-apoptotic activity. These results suggest that the protective effect of AGP is likely mediated by its sialic acid terminal groups.

Keywords: Apoptosis; Acute phase response; Bovine; Monocytes; α1-Acid glycoproein; Glycosylation

1. Introduction

α_1-Acid glycoprotein (AGP, orosomucoid) is an acute phase protein produced mainly by hepatocytes, but also by other cellular types, in response to systemic infection or injury (Hochepied et al., 2003). From a structural and functional point of view, AGP belongs to a sub-group of binding proteins known as immunocalins, a lipocalin sub-family that is able to exert significant immunomodulatory effects (Logdberg and Wester, 2000). The precise nature of AGP function is still unknown. However, it features at least two different biological activities, apparently very different from each other. AGP may act as a serum binding protein, and at the same time regulate the inflammatory response of blood white cells (Hochepied et al., 2003)(Fournier et al., 2000). All the various immunomodulatory activities of AGP described so far converge toward an overall ameliorative function, that consists in downgrading of the inflammatory status with the aim of reducing collateral damage caused by inflammation. One of the several protective features of AGP activity is its ability to protect cells from apoptosis. It has been

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shown that AGP can prevent apoptosis in liver cells after an in vivo challenge with TNFα and actynomycin D (Van Molle et al., 1997), probably by preventing the activation of caspases-3 and caspase-7 (Van Molle et al., 1999). A similar protective effect has been reported in murine model of renal and ischemia-reperfusion (Daemen et al., 2000; De Vries et al., 2004). The rate of apoptosis can be viewed as a way to control the activity of blood granulocytes and monocytes, either by reducing or increasing their lifespan in inflamed tissues.

Since AGP may promote an increase in the expression of anti-inflammatory cytokines by macrophages, it can be hypothesized that AGP may increase the lifespan of cells that produce anti-inflammatory molecules. Very few data are availables about boAGP's biological activity on bovine blood cells. Bovine AGP (boAGP) is considered a minor acute phase protein in cattle, its concentration increasing from two to four fold during disease (Tamura et al., 1989; Eckersall et al., 2001). Mature boAGP is expressed as a single polypeptide chain of 20.4 kDa, the carbohydrate moiety accounting for 40% of total mass (Ceciliani et al., 2005; Nakano et al., 2004). To the authors's knowledge, there are no reports on ability of bovine AGP, or of any other other species, to regulate the apoptosis of monocytes. The first aim of the present study was therefore to verify if AGP purified from bovine blood can reduce the apoptosis rate of one of the defensive cells involved in the inflammatory process, the blood derived monocyte. The second point that we wanted to address in our investigation was the involvement of the carbohydrate moiety in the apoptosis-modulating activity of boAGP. Hyperfucosylated AGP molecules have been shown to be protective against experimental ischemic/reperfusion apoptosis model more efficiently that non-fucosylated AGP (Williams et al., 1997). Furthermore, desialylated AGP did not show a hepatoprotective effect in a rat-hepatitis model (Kagaya et al., 2005). This data would suggest that the carbohydrate component may contribute to the ''ameliorative'' activity of AGP. In the second part of the esperiments we therefore investigated whether the apoptosis-modulating activity of boAGP was dependent on the terminal sialic acid residues exposed on the surface of the protein.

2. Materials and methods

2.1. Purification of bovine AGP

Purification of bovine AGP was carried out following some modifications of a previously described method (Ceciliani et al., 2005). Briefly, 200 ml of bovine serum was dialysed overnight against a buffer citrate-phosphate 10 mM, pH 4.0, centrifuged at 14,000 × *g* × 5 min and the supernatant was loaded onto a HiTrap Q Sepharose XL (16/25 mm) (Amersham Biosciences). The fractions containing boAGP were eluted with buffer citrate-phosphate 100 mM, pH 4.0. Positive fractions were pooled and concentrated to 10 ml using Centricon 30 (Millipore). Meanwhile, citrate-phosphate buffer 100 mM was changed to sodium phosphate buffer, 10 mM, pH 7.6. The proteins were loaded onto a Heparin Affinity column (Amersham Biosciences) previously equilibrated in Tris, 10 mM, pH 7.6. BoAGP is not retained by the column and is eluted in the void volume. Fractions containing boAGP were directly loaded onto a Sephasil Protein C4 (5 μm ST 4.6/100 mm) cromathographic column (Amersham Biosciences) equilibrated with 0.065% TFA (Trifluoroacetic acid) in water. Protein separation was carried out using a 20%–100% gradient of acetonitrile +0.05% TFA over 30 min, at a flow rate of 1 ml/min. The fraction containing boAGP, which was eluted after approximately 15 volumes, was collected and used for further analysis. The boAGP protein content was determined either by direct spectrophotometric measurement at 280 nm and by the specific radial immuno diffusion assay for bovine AGP (Bovine a1AG Plate, Tridelta Development Ltd). Aliquots of 1mg of purified boAGP were dried to 50 μl in Savant microconcentrator apparatus in order to remove TFA and Acetonitrile residues: 500 μl of endotoxin free water (Sigma) was added, and brought again to 50 μl. This step was repeated for three times, and the protein was finally resuspended in 50 μl of RPMI-1640, 20 mM Hepes, 10% FCS. The endotoxin content of the boAGP used for these experiments was assessed by Limulus amoebocyte lysate assay (LAL test) (BioWhittaker).

2.2. Production of polyclonal anti-boAGP antibody

A polyclonal antibody anti-bovine AGP was raised in rabbits following standard immunization procedures and using 2 mg of boAGP purified as previously described. Commercial boAGP (Sigma) was used to test the reactivity of the polyclonal antibody by Western blotting: 100 ng of the protein were analysed onto 12% SDS–PAGE and Western blotted onto nitrocellulose membrane. Nitrocellulose blots were blocked for 20 min with 2% (w/v) milk powder in PBS, 0.1% Tween at room temperature. The membranes were then incubated with different concentrations of polyclonal anti-boAGP primary antibody (1:1000, 1:2000, 1:5000 and 1:10,000) diluted in PBS, 0.1% Tween, for 1hr at room