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TESI DI DOTTORATO DI RICERCA

## **AGP regulates the neutrophil and endothelial cell inflammatory response.**

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*A la Mercè, que no ho va dubtar mai.*

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## **Aims of this thesis**

Neutrophil degranulation, angiogenesis and wound healing are three important host responses during inflammation. In this thesis we wanted to study how the immunocalin  $\alpha$ 1-acid glycoprotein (AGP), in its role of immunomodulatory acute phase protein, affected these processes in order to help get a better overall comprehension of the acute phase reaction. To do so we studied bovine neutrophil degranulation within the frame of acute inflammation and, on the lack of a good bovine endothelial model, we studied angiogenesis and wound healing on endothelial human primary cells.

## **Acute Phase Proteins and the Bovine Mammary Gland**

During mastitis the bovine mammary gland produces a bunch of proteins to defend itself from a bacterial attack or injury. The so called acute phase proteins (APP). These proteins exhort several immunological functions:

- Opsonization
- Recruitment of immunological cells (neutrophils and monocytes)
- Induction of metalloproteases (MPP) to degrade the extracellular matrix
- Complement activation
- Inhibition of the coagulation
- Iron binding
- Hemoglobin binding

Its production and action needs to be tightly regulated to minimize and dampen the side effects of inflammation. When the local production of APP is not sufficient to contain or eradicate the infection (local response), the liver gets in action, producing a much more efficient systemic response (Rainard 2005).

The bovine mammary gland during its local response produces different APP and other immune-related factors with diverse activities all of them focused on fighting against the invading bacteria (Rainard&Riollet 2006).

The **lipopolysaccharide-binding protein (LBP)** is one of them, its classic function is to bind LPS, to present it to CD14 cells and activate the toll like receptor 4 (TLR4) pathway (Zweigner 2006) though now we know that it also recognizes other pathogen associated molecular patterns (PAMP's) a group of cytoplasmatic molecules anchored to the membrane of Gram-positive bacteria and activating the TLR2 pathway, making of LBP a crucial APP since it can amplify the immune response against both classes of bacteria (Rahman 2010). Plasma levels can increase up to seven fold during experimental infection with

*Mannheimia haemolytica* (Pasteurella) (Schroedl 2001), and both plasma and milk levels do increase during experimental mastitis (Bannerman 2003).

It is mainly synthesized by the hepatocytes but extra hepatic expression has been reported (Rahman 2010) in several bovine tissues with different intensity and distribution:

<b>Tissue</b>	<b>Intensity</b>	<b>Distribution</b>
Lung	Low	Multifocal
Mammary gland	High	Diffuse
Parenchyma	High	Diffuse
Cistern	Moderate	Diffuse
Teat	High	Diffuse

Extra hepatic expression of LBP in bovine tissues in physiological conditions (Modified from Rahman 2010)

**Serum Amyloid A3 (SAA3)** is one of the major reactants during the acute inflammation response (Molenaar 2009). It can increase up to 1000-fold in human and mice whereas it's levels in bovine milk can rise up to 110µg/ml during mastitis (Eckersall 2001). As with many other APP it's exact role it not yet fully understood but it's multifunctional and might include several immunological functions (Jensen&Whitehead 1998).

In humans different SAA's mRNA's have been detected in different tissues including intestine, pancreas and breast epithelial cells (Urieli-Shoval 1998). In the bovine mammary gland it was found at low levels in epithelium with lactation phenotype but at high levels in ductal epithelial cells and vesicle engorged alveoli (Moleenaar 1996) and strongly induced during mastitis in secretory mammary epithelial cells (MEC). With the exception of the liver SAA3 expression profile during different physiological states it's very similar to that found for lactoferrin (Molenaar 1996) being more expressed during pregnancy, involution and infection than during lactation.

Bovine milk contains very little **Lactoferrin (Lf)** 20-200ug/ml compared with human mil 1-2mg/ml (Rainard 2006). The main source of Lf in bovine milk are the mammary epithelial cells (MEC), and while it's expression it's moderate/high

in the ducts and cisterns it's totally absent in the proximal end of the teat canal (Molenaar 1996). Main function of Lf is bacteriostasis through its iron-chelating properties, *E.Coli* being the most susceptible to its action among the mastitis-causing bacteria since it has high iron requirements. These iron-binding properties are probably of low efficiency during lactation but likely to be significant when the mammary gland is fully involuted (Rainard 2006). Lf also activates the classical complement pathway resulting in opsonization of the bacteria as it has been shown in *S.agalactiae* (Rainard 1993). It also seems to have an overall dampening action during the acute mastitis by binding to LPS (lipopolysaccharide a constituent of gram-negative bacteria), LTA (lipoteichoic acid a major constituent of gram-positive bacteria) and CpG (Rainard 2006).

**Haptoglobin (Hp)** is also mainly secreted by liver, it is a major APP in ruminants (Hiss 2004) and has been suggested and used as a diagnostic marker for mastitis (Eckersall 2001) and respiratory diseases in cattle (Heegaard 2000). The use of milk Hp as an early marker for mastitis does not work due to the low detection limit of the assay (Hiss 2004) but the fact that Hp can be found in milk during experimental LPS provoked mastitis even before it increases its serum concentration demonstrates the fact that mammary gland synthesizes its own local Hp which might be used for its bacteriostatic properties (Eaton 1982, Hiss 2004) its down regulation of the immune response by suppressing production of proinflammatory cytokines in monocytes, or its capacity to inhibit the respiratory burst activity in neutrophils.

**$\alpha$ 1-Acid glycoprotein (AGP, Orosomucoid)** is a minor acute phase protein in cattle (Tamura 1989) where it can rise up to 3 fold during acute phase inflammation rising from physiological levels (0.3mg/ml) to acute phase levels (0.9mg/ml) it is mainly produced by liver but extrahepatic expression has been widely reported in other human tissues such as stimulated alveolar macrophages (Fournier 1999), breast epithelial cells (Gendler 1982) or endothelial cells (Sorensson 1999) as well as in several bovine tissues (Lecchi 2010) like spleen, sub-mandibular gland, lung, uterus and pancreas with of course a higher mRNA

expression found in liver, and in at a much lower level in other tissues as lymph node, parotid gland, tongue or kidney. In all of them the immunohistochemistry revealed endothelial expression, except for liver where AGP was found all over the cytoplasm. In the mammary gland AGP was found in the epithelium (Ceciliani 2005) where it probably has an important role dampening the collateral effects of the acute inflammation during mastitis that are often responsible for severe lesions to the mammary epithelium (Burvenich 2003). As is Hp and SAA, AGP is also present in milk during mastitis where it can be found in two forms a low MW (44kDa) isoform that is expressed by the mammary gland epithelium (Ceciliani 2007) and a much less abundant high MW one (55-70kDa) produced by somatic cells (SC), that considerably rises its concentration during mastitis when large amounts of polymorphonuclear cells (PMNC) pass thorough the hemato-mammary barrier to get to the local site of inflammation and release its granules. This high MW AGP is apparently identical to the isoform found in PMNC which in humans has been reported to be expressed during the mieloproliferative phase and then carried to the inflammation focus by the mature PMNC (Theilgaard-Monch 2005). This uncontrolled influx of activated PMNC to the mammary gland is considered to be the major cause of damage to the mammary gland during mastitis (Ceciliani 2007) the release of the SC-AGP at the local inflammation spot where it exerts all its anti-inflammatory activities, including dampening of the inflammation and increasing the bacterial resistance is a very important process that still needs to be fully understood.

## **The $\alpha$ 1-acid glycoprotein (AGP)**

$\alpha$ 1-acid glycoprotein (AGP), also known as orosomucoid (ORM) was first described by Karl Schmid and Richard J. Winzler in 1950 (Schmid 1950, Weimer 1950). It is one of the major positive acute phase protein (APP)s in the human body (Fournier 2000) and in several different species (Ceciliani 2007). Throughout evolution, AGP has been highly conserved, and is basally expressed over the life span, even in the face of severe starvation (Liao 1985). Although many important biological activities have been attributed to AGP, its exact physiological function(s) remains unclear. AGP is found in plasma under physiological conditions; its concentration in the serum/plasma can increase several fold during the acute phase response.

	<b>concentration in plasma (mg/ml)</b>	<b>Increase during acute phase reaction</b>
<b>Human</b>	<b>0.5-1</b>	<b>I</b>
<b>Bovine</b>	<b>0.2-0.45</b>	<b>I</b>
<b>Pig</b>	<b>0.3-0.56</b>	<b>O/I</b>
<b>Dog</b>	<b>0.05-0.8</b>	<b>I</b>
<b>Cat</b>	<b>0.1-0.48</b>	<b>I</b>
<b>Mouse</b>	<b>0.2-0.4</b>	<b>I</b>
<b>Rat</b>	<b>0.1-0.2</b>	<b>II</b>

**AGP PLASMA LEVELS IN MULTIPLE SPECIES.** O: no change, I: 1-10-fold increase, II: >10 fold increase. (Ceciliani 2007).

AGP has very unusual biochemical characteristics. It has a molecular weight of 41-43kDa, with a high carbohydrate content of 45% (Fournier 2000, Schmid 1977). This molecule has an extremely low pI of 2.8-3.8 and an unusually high solubility in both H<sub>2</sub>O and in polar organic solvents (Fournier 2000).

### **Amino Acid Core of AGP**

AGP is encoded by 3 adjacent genes in chromosome 9. The predominant gene product, AGP-A (ORM1) is upregulated during the acute phase response, encodes a 201 amino acid (aa) precursor protein with a secretory NH<sub>2</sub>-terminal signal peptide of 18 residues (Schmid 1973, Herve 1998, Schonfeld 2008).

## AMINO ACID SEQUENCE FOR AGP IN MULTIPLE SPECIES

```

MOUSE      MALHTVLIILSLLPMLEAQNPESHANFTIGEPITNETLSWLSKWFYMGAAFRKLEYRQAI 60
RAT        MALHMLVVLVLSLLPLLEAQNPANITLGIPITNETLKWLSKWFYMGAAFRDPVFKQAV 60
HUMAN      MALSWVLTVLSLLPLLEAQIPLCANLVPVP-ITNATLDQITGKWFYIASAFRNEEYNKSV 59
BOVINE     MALLWALAVLSLLPLLDASPECANLMTVAPITNATMDLLSGKWFYIGSAFRNPEYNKSA 60
          ***  . * :*****:*** * **:      *** *. . :.:*****:..***. :.:

MOUSE      QTMQSEFFYLTTNLINDTIELRESQTIGDQCVYNSTHLGFQRENGTFSKYEGGVETFAHL 120
RAT        QTIQTEYFYLTNPNLINDTIELREFQTDDQCVYNFTHLGVQRENGTLSKCAGAVKIFAHL 120
HUMAN      QEIQATFFYFTPNKTEDTIFLREYQTRDQCIYNTTYLNVQRENGTISRYVGGQEHFAHL 119
BOVINE     RAIQA AFFYLEPRHAEDKLITREYQTIEDKCVYNCSFIKIYRQNGTLSKVESDREHFVDL 120
          : :.: :***: . . :.: ** ** *:***: .: . *.:***:*. : : *..*

MOUSE      IVLRKHGAFMLAFDLKDEKKRGLSLYAKRPDITPELREVFQKAVTHVGMDESEIIFVDWK 180
RAT        IVLKKHGTFMFAFNLTDEN-RGLSFYAKKPDLSPELRKIFQQAVKDVGMDESEIIFVDWT 179
HUMAN      LILRDTKTYMLAFDVNDEKNWGLSVYADKPETTKEQLGFEFYEALDCLRIPKSDVVYTDWK 179
BOVINE     LLSKHFRTFMLAASWNGTKNVGSFYADKPEVTQEQQKEFLDVIKICIGIQESEIIYTDEK 180
          :. :. :***** . . : *:*..*.:* : * * ..: : : :*****.* .

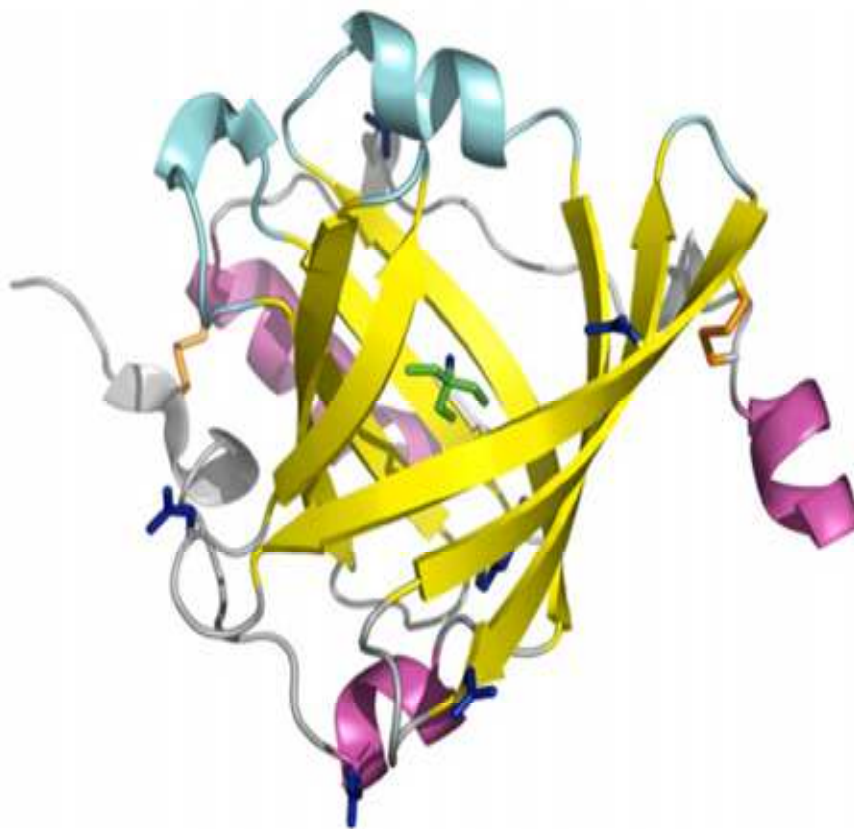
MOUSE      KDRCGQQEKKQLELGKETKKDPEEGQA 207
RAT        KDKCSEQQKQLELEKETKKETKDP- 205
HUMAN      KDKCEPLEKQH--EKERKQEEGES-- 201
BOVINE     KDACGPLEKQH--EERKKETEAS-- 202
          ** * :.: : * *.: .

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Amino acid sequence alignment and homology comparison of AGP's primary structures of  $\alpha$ 1-acid glycoprotein from different species. The accession numbers (Swiss-Prot) are the following: mouse Q60590, rat P02764, human P02763, bovine AJ844606.

AGP is comprised of a single polypeptide chain of 201-207 residues, depending on the species, two disulphide bridges linking cysteine pairs 5-147 and 72-165 (Schmid 1974) which play a crucial role for its correct folding and intramolecular and intermolecular interactions (Nishi 2006).

The aa backbone of human AGP has been resolved to 1.8 Å and was found to fold in a typical lipocalin tertiary conformation (Schonfeld 2008). This structure is comprised of 1) an eight stranded beta-barrel as a central folding motif, 2) a flanking  $\alpha$ -helix, and 3) four loops connecting the  $\beta$ -strands to form the entrance to a ligand-binding pocket at the open end of the  $\beta$ -barrel.



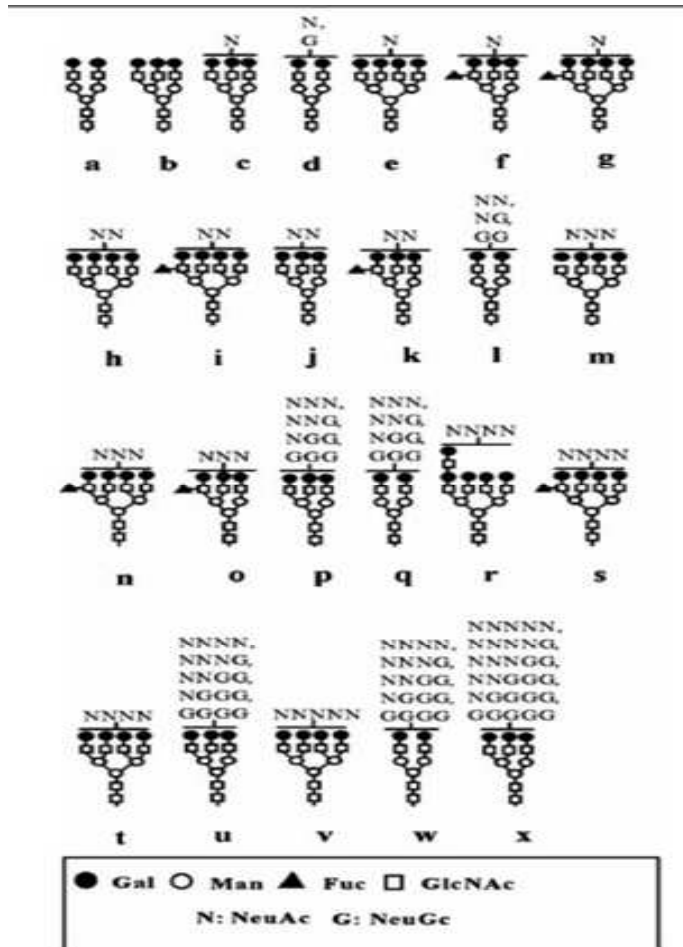
The refined crystal structure of human AGP at 1.8Å resolution shown as a ribbon cartoon in stereo view. Secondary structure is colored yellow ( $\beta$ -strands), pink ( $\alpha$ -helix), and gray (coils), while the two disulphide bridges are shown in orange. The open end of the  $\beta$ -barrel is highlighted in cyan. The 5 N-glycosylation sites and the residue Trp25 (conserved throughout the lipocalin family) are depicted as blue sticks. (Schonfeld 2008).

Earlier studies indicate that AGP has a wide and flexible drug-binding area to which both acidic and basic drugs can bind with considerable overlap (Maruyama 1990). In fact, the nonglycosylated AGP presents a cleft-like cavity that measures 9-12Å in diameter, is ~18Å deep, and contains three distinct lobes, a main deep nonpolar lobe flanked by two smaller negatively-charged lobes (Schonfeld 2008). The intricate geometry of the cavity and the flexibility of its structure, explains the broad spectrum of basic and neutral compounds that can be engaged by this lipocalin (Schonfeld 2008, Israili 2001).



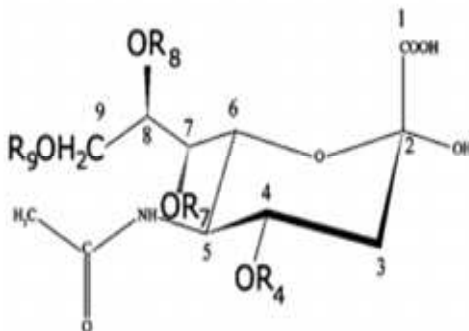
## Glycosylation

AGP is heavily glycosylated. Approximately 45% of its 43kDa molecular mass can be attributed to attached sugar chains, presenting 5 N-glycosylation sites (Asn-15, -38, -54, -75, -85) that are not conserved across species lines. These glycans contain a wide range of sialo-oligosaccharides, being one of the few serum glycoproteins that can express di-, tri- and tetra-antennary N-linked glycans (Fournier 2000, Ceciliani 2007).



**List of sialo-oligosaccharide structures found in human, bovine, and rat AGP.** (Ceciliani 2007) N indicates N-acetylneuraminic acid (NeuAc). G indicates N-glycolylneuraminic acid (NeuGc). For detailed description of the structures refer to the paper of Dr. Nakano 2004. (Courtesy of Dr. Nakano)

Of these glycans, 10-12% are terminated by a sialic acid residue (NeuAc), each coupled through  $\alpha(2-3)$  or  $\alpha(2-6)$  linkages to a subterminal galactose residue. This high sialylation state is responsible for the low pI (2.8-3.8) of AGP (Ceciliani 2007).



**Structure of N-acetylneuraminic acid (Neu5Ac)**, the main representative of the sialic acid family, and some of its modifications (Lamari 2002) R4: acetyl, R7: acetyl, R8: acetyl, methyl, sulfate, R9: acetyl, phosphate, lactoyl. (Ceciliani 2007)

Such a high diversity of glycan expression would predict more than  $10^5$  distinct glycoforms of AGP. However, some combinations have not been described in nature. In fact, only 12-20 different glycoforms can be detected in healthy human serum (Ceciliani 2007, Treuheit 1992). The glycosylation pattern is strongly influenced by the pathophysiological condition of the host, including acute inflammation, pregnancy, severe rheumatoid arthritis, and alcoholic liver cirrhosis or hepatitis (Fournier 2000, Biou 1991, Jezequel 1988, Biou 1989, Serbource-GoguelSeta 1986, Wieruszeski 1988). The glycosylation site Asn-38 is located in the  $\alpha$ -helix. A change in its glycosylation, might influence the tertiary conformation of the molecule and thus, its ligand-binding activity (Ceciliani 2007).

In addition to its 5 N-glycosylation sites, AGP also contains 8 potential phosphorylation sites, that vary in number and position depending on the species (Ceciliani 2007). Surprisingly, very little is known about the phosphorylation state of AGP and whether it regulates its biological function.

## Gene Structure

Human AGP is encoded in chromosome 9, in a 70kb region, encoded by 3 adjacent genes: AGP-A, AGP-B, AGP-B'.

AGP-A is the major gene product expressed in liver, about 100-fold more than the other two AGP isoforms. AGP-B and AGP-B' are identical and each only differs from AGP-A by 22 base substitutions (Fournier 2000). These two genes are probably a result of a recent duplication (Dente 1987).

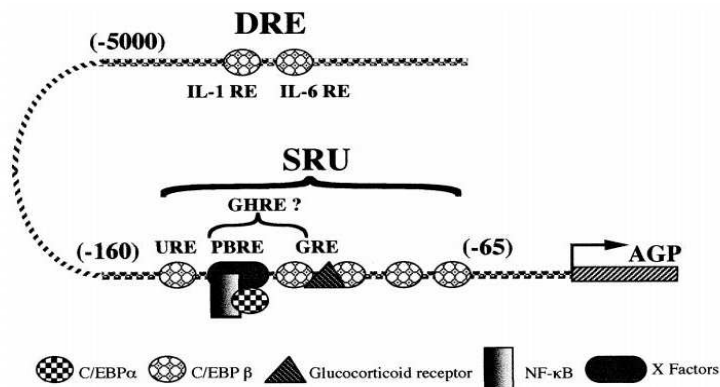
AGP-A gene is constructed of six exons and five introns. The whole coding region is included in a 4.6kb HindIII fragment (Dente 1987) that includes the coding region plus a 5' and a 3'-flanking DNA region that are 1.2kb and 2kb long, respectively (Dente 1988).

The cDNA is 823bp long, with 78 untranslated bases at the 5' end and 143 untranslated bases at the 3' end, adjacent to the polyA tail (Dente 1985).

Rat AGP is encoded by a single gene (Liao 1985).

*Mus caroli* has 8 genes encoding AGP (Prowse 1990). However only two of them are expressed at the protein level.

BALB/c mice express two forms of AGP, AGP-1 and AGP-2, each encoded in chromosome 4 (Cooper 1986). Mice, as is true for rats, have the same gene structure for AGP as do humans, six exons and five introns (Fournier 2000).



### **Regulatory elements and trans acting factors implicated in the expression of the AGP gene.**

The AGP gene promoter contains several positive cis-acting sequences within the SRU that are involved in its regulation by glucocorticoids: the GRE that binds the glucocorticoid receptor

interacting with C/EBP $\beta$ ; the upstream responsive elements (URE) and other regions that interact with C/EBP $\beta$ . All these elements are essential for maximal induction of the AGP promoter by glucocorticoids. The PBRE and the region probably involved in growth hormone response are located within the SRU, and interact with unknown factors (X factor), NF-kB and C/EBP $\alpha$  factors. The distal responsive element (DRE) implicated in the regulation of AGP by cytokines is located in the enhancer region and interacts with C/EBP $\beta$ . (Fournier 2000).

AGP is a positive APP, and like serum amyloid A, haptoglobin and C-reactive protein, is regulated by the pro-inflammatory cytokines, IL-1, IL-6, and IL-8 as well as by glucocorticoids (Fournier 2000). Retinoic acid can also indirectly modulate hepatic expression of AGP through increased IL-6 biosynthesis (Ceciliani 2007).

During the acute phase response, the serum concentration of AGP not only increases but its glycosylation pattern changes. Its sialylation and fucosylation states both change during an inflammatory process (Shiyan 1997) and these two modifications are independently regulated from its protein synthesis (DeGraaf 1994,1995).

Terminal sialic acids on AGP influence its half-life. Desialylation of AGP decreases its  $t_{1/2}$  from 5 days to 2 minutes (Weis 1989).

### **AGP as a Binding and Transportation Protein**

AGP is one of the most abundant proteins in plasma (Schmid 1975). And together with albumin and the lipoproteins is one of the most important binding proteins in plasma. Its 3D structure, rich in  $\beta$ -sheets clearly resembles the conformation of a transport protein, the so called lipocalin signature (Schonfeld 2008). It has been demonstrated that in non pathological circumstances AGP can bind more than 300 different molecules and drugs (Israili 2001). The binding and transport of some ligands, specially endogenous ones are probably involved in several important biological activities of the protein (Ceciliani 2007).

Plasma AGP's concentration is much lower than albumin in physiological conditions, but during the acute phase response AGP becomes one of the most important binding/transporting protein for endogenous molecules since its

concentration is several fold increased while albumin's, being a negative acute phase protein, decreases. In fact, during analbuminemia, AGP plays a major compensatory role by binding many amphipathic mediators becoming their principal carrier in the absence of albumin (Israili 2001).

Due to its very special physical-chemical properties (pI 2.8-3.8) AGP can bind basic and neutral molecules. AGP can also bind endogenous ligands, such as heparin, IgG3, serotonin (Schmid 1972), platelet activating factor (PAF) (McNamara 1986), melatonin (Morin 1997), histamine (Chachaj 1980), and steroid hormones (McPherson 1980). Interaction of AGP with amphipathic mediators of inflammation such as PAF or PgE2 has also been reported (Ivanov 2005). AGP is also the main vanilloids-binding protein in serum (Szallasi 1992) which can be inhibited by chlorpromazine and trisbutoxy-ethylphosphate indicating that AGP presents a specific binding domain for vanilloids. AGP can also bind acidic molecules like retinoic acid (Israili 2001), phenobarbital (Schley 1983) and propranolol (Albani 1984) which is bound with different stereospecific affinity, being (-)propranolol much easily bounded than (+)propranolol. Some other compounds have been found to have several binding sites, like estradiol, which can bind to 7 different sites (Kerkay 1968).

Some of the molecules binded and carried by AGP also regulate its own expression (Ceciliani 2007). Retinoic acid is able to increase AGP gene expression in rat hepatocytes (Mouthiers 2004), whereas phenobarbital induces AGP gene overexpression (Mejdoubi 1999). AGP can also bind to toxic molecules produced by microorganisms such as toxic lectins (Frantz 2000) and bacterial lipopolysaccharide (LPS) (Libert 1994), thus serving as a general protective agent against septic shock.

Endothelial cells constitutively produce AGP and export it to the glycocalyx where it plays an important role in maintaining capillar permeability (Haraldsson 1987) where in rodent models has been shown to exhort a protective function by maintaining organ perfusions (Muchitsch 1998).

AGP can also bind other serum proteins. In particular, AGP binds plasminogen activator inhibitor type 1 (PAI-1) (Boncela 2001). PAI-1 is a serpin (a serin protease inhibitor) and plays a very important role in the regulation of fibrinolysis.

(Ceciliani 2007). It is usually complexed with vitronectin and circulates in this form (Declerck 1988). AGP has a much lower affinity for PAI-1 than vitronectin, but during the acute phase its concentration can be up to ten-fold higher (Berk 1990) suggesting that AGP might act as a reservoir of the biologically active form of the protease inhibitor, specially during the acute phase. Moreover, during the acute phase, the complex AGP/PAI-1 may accumulate on the surface of the endothelium due to the high affinity of its glycan moiety to E-selectin on the endothelial cell surface, inducing a local generation of thrombin at the inflammation site (Ceciliani 2007). AGP as other lipocalins, is also involved in the transport of olfactory substances (Guiraudie 2003) in the nasal mucosa through the binding of lauric acid.

## **NEUTROPHILS**

Neutrophils are the first line of defense against diverse forms of injury and invading bacteria. These cells arrive to the site of infection within minutes to neutralize the threat through multiple mechanisms. Neutrophils phagocytose bacteria, produce reactive oxygen intermediates (ROI), and release antimicrobial contents of their granules (Borregaard 1997). Human neutrophils have secretory vesicles and primary (azurophilic) and secondary (specific) granules that contain antimicrobial proteins and proteases that, upon activation, are released at the site of tissue injury. Small ruminants and cows also have a unique, large type of granules that are not shared by humans (Gennaro 1983). Whereas the contents of both granules and secretory vesicles have been biochemically characterized, their mobilization and regulation are poorly understood. Contents of those granules are cytotoxic and essential to oppose infections but at the same time failure to control their release can provoke massive damage to the surrounding tissues (Smith 1994).

In both human and bovine species, two distinct isoforms of AGP can be found. One highly glycosylated AGP isoform has a high MW (50-60 kDa) and is

synthesized in myeloid cells in the bone marrow where they are and stored in secondary granules (Theilgaard-Monch 2005). Another far less abundant AGP isoform has a much lower MW (42-45 kDa) and after its release from the liver is endocytosed by neutrophils where it is stored in secretory vesicles (Rahman 2008).

Upon neutrophil activation, secretory vesicles are the very first ones to be released, readily followed by secondary granules. This pattern of release indicates that AGP exerts both local and systemic immunomodulatory activity (Fournier 2000). Even if the amount of AGP released by neutrophils at the inflammatory site might seem “not important” compared to the plasma AGP we can’t exclude its relevance since it’s known that as little as 1µg/ml of AGP increases the cytosolic  $\text{Ca}^{2+}$  in humans (Gunnarsson 2007).

The distinct molecular weights of the two isoforms found in neutrophils are due to different post-translational modifications which suggests that glycosylation does not only depend on the physiological state of the host but also on the tissue that expresses the protein (Rahman 2008).

AGP is not only stored in neutrophils it also binds to its membranes regulating different activities. As little as 1µg/ml of AGP rises neutrophil cytosolic  $[\text{Ca}^{2+}]$  in humans (Gunnarsson 2007) and acute phase concentrations (0.9mg/ml) diminish exocytosis of specific granules in bovine (Miranda-Ribera 2010).

## **THE ENDOTHELIUM**

The microvascular endothelium presents a selective barrier that actively regulates movement of circulating fluid, macromolecules, and cells into extravascular tissues and compartments. Several distinct intercellular junctions regulate the paracellular movement of high molecular weight proteins, lipids and cellular mediators of the immune system. While some endothelia like the cerebral

microvasculature express predominantly tight junctions, other endothelia like the hepatic endothelium contain much larger gaps permitting the passage of larger molecules. Since this heterogeneity not only involves cell junctions, but also surface antigen expression and growth factor responsiveness, it is crucial to study each distinct type of endothelium (Prudence 1993).

AGP plays a vital role maintaining normal endothelial barrier integrity in rat skeletal muscle (Haraldsson 1987). Similar results were shown in frog mesenteric capillaries (Curry 1989). In both cases, AGP was required to maintain the capillary charge barrier, due to its low pI of 2.7 rather than a change in the equivalent pore radius since the hydraulic conductivity never changed. These same findings were extended to the very specialized structure of the glomerulus (Haraldsson 1992) where AGP was needed to maintain normal barrier function. Moreover, dermal human endothelial cells synthesize their own AGP (Sorensson 1999) to help maintain their own intrinsic charge selectivity. The interaction of AGP with the endothelial glycocalyx has not been fully studied. However, AGP is known to affect the 3D arrangement of collagen and bovine serum AGP (boAGP) binds to the surface of bovine pulmonary microvascular endothelial cells (BLMVEC) (Schnitzer 1992) in a ligand-receptor manner even if the receptor has not yet been defined.



**AGP IN NEUTROPHIL GRANULES AND ITS EFFECT**  
**ON DEGRANULATION**

## **Introduction**

Innate immunity strongly relies on the activity of polymorphonuclear (PMN) neutrophil leukocytes. They represent the first line of defence against invading pathogens by migrating to sites of infection within minutes from any injury, in order to search and destroy foreign intruders (Smith 1994). Neutrophils fulfil their role mainly by internalizing and exposing pathogens to the destructive action of reactive oxygen species (ROS) and hydrolyzing enzymes stored in their granules (Roos 2003). Bovine neutrophils, like other species, contain secretory vesicles, primary (azurophilic) and secondary (specific) granules. In addition, bovine and other small ruminants, such as goats and sheep, share unique, large, granules that are not present in human cells (Gennaro 1983, Paape 2003). While the organization of granules in bovine neutrophils is known, there is a relative lack of knowledge about the molecules which modulate their mobilization. Neutrophil exocytosis occurs due to the translocation of granules. This process is hierarchical, secondary granules being mobilized more readily than primary ones, in humans as well as in bovines (Watson 1995, Borregaard 1997, Paape 2003), and it is likely to be dependent on specific remodelling events. Due to their very high destructive potential, both PMN and organism as a whole have developed tight controlled mechanisms whose role is down regulating and limiting the destruction of surrounding cells and tissues (Serhan 2005). The resolution of the neutrophil phase of inflammation depends on the fine tuning of their lifespan, regulated by apoptosis, but also by the presence of molecules that may act as down regulators of the PMN action. The immunocalin  $\alpha_1$ -acid glycoprotein (AGP) possesses an immunomodulatory activity and it is believed to play an important role in the regulation of local inflammatory reaction, for example by reducing the tissue damages caused by an excessive activation of complement (Tilg 1993). The concentration of AGP rises in plasma from three to five folds during systemic reaction of inflammation (Hochebied 2003, Petersen 2004), and therefore is considered, at least from a clinical perspective, a minor acute phase protein. AGP is mainly synthesized by liver, but it can be localized in several other human (Hochebied 2003) and bovine (Lecchi 2008) tissues. AGP exerts a sort of

protective activity by reducing the apoptosis rate in some inflamed tissues (Van Molle 1997) and by increasing the lifespan of monocytes, at least in the bovine species (Ceciliani 2007). Defensive functions of neutrophils can be affected as well: for example, AGP has been reported to influence neutrophil chemotaxis (Laine 1990, Vasson 1994), aggregation and generation of reactive oxygen species (Costello 1984). The activity of AGP on neutrophil exocytosis has not been investigated so far, neither in human nor in veterinary medicine. Our hypothesis is that, due to its well known immunomodulatory function, AGP acts as a local regulatory mechanism that modulates the exocytosis of granules from bovine neutrophils after their recruitment in the inflammatory focus. Therefore, the major aim of this study was to investigate the capability of purified bovine AGP to influence the degranulation of neutrophils by studying the possible relationship between AGP and the exocytosis of primary and secondary granules. The hypothesis that AGP may modulate neutrophil degranulation by competing with inflammatory mediator receptors, which in our experimental design included ZAS, was also explored. Finally, since AGP is one of the most glycosylated protein in the organism, and its glycan pattern strongly influences its biological activity [3], in the last part of this study the relationship between the terminal sialic acid residues of AGP and its exocytosis-modulating activity was investigated by using in parallel a desialylated glycoform.

## **Materials and Methods**

### *Reagents*

All reagents used in these experiments were purchased from Sigma-Chemicals Co., unless otherwise specified. Hanks balanced saline solution with 0.5 mM  $\text{CaCl}_2$  and 1mM  $\text{MgCl}_2$  (HBSS+) was used throughout all the experiments. NaCl solutions were diluted starting from sterile cell tested 5M NaCl. Cell culture tested, endotoxin free, albumin was purchased from GIBCO (Invitrogen S.R.L. – Milano, Italy). Bovine serum was activated with 15 mg/ml of Zymosan A from *S. cerevisiae* at 37°C for 60 min. The mixture was then incubated at 56°C for 30 minutes to destroy complement components with the exception of C5a. Zymosan

particles were removed by centrifuging the mixture for 15 minutes at 1000 x g at 4°C. The obtained supernatant was filtered through a 0.22µm filter membrane (Millipore, Segrate, Italy), stored at -80°C and used within three months. Degranulation was induced using the ZAS stock solution diluted 1:5 vol/vol with HBSS+. All the experiments carried out in this study used bovine AGP aliquots purified from plasma as previously reported (ceciliani 2007). Two different concentrations of purified AGP were used: low concentration, similar to that physiologically found in bovine plasma of healthy subjects (0.3 mg/ml) and high concentration, similar to that found in bovine plasma during acute phase response to several inflammatory statuses (0.9 mg/ml) (Tamura 1989).

#### *Bovine neutrophils isolation*

Clinically healthy lactating Holstein cows between 2 and 7 years of age were used throughout these studies as blood donors for all experiments. Blood was obtained from the jugular vein and collected into blood bag containing acid-citrate-dextrose (Terumo, Belgium). Samples containing less than 5% eosinophils were used. Neutrophils were isolated using a Percoll®-gradient as previously described (Rinaldi 2007), with slight modification. Briefly, 40 ml of blood were transferred to 50 ml polypropylene conical tubes and centrifuged (1000 × g) for 20 minutes at 4 °C. The plasma and buffy coat were aseptically aspirated and discarded. The remaining cells were suspended in 35 ml final volume of ice-cold PBS and the suspension slowly pipetted down the side of a clean 50 ml polypropylene conical tube containing 10 ml of 1.087 g/ml Percoll®. Samples were centrifuged (400 × g) for 40 minutes at 20°C. The supernatant, mononuclear cell layer, and Percoll® were aseptically aspirated and a pellet composed of PMN and erythrocytes was retained. Erythrocytes were lysed by mixing 1 volume of cells with 2 volumes of an ice cold 0.2% NaCl solution and inverting the tube for 1 minute. Tonicity was restored by the addition of one-half volume of a 3.7% NaCl solution. The samples were centrifuged at 500 × g for 2 minute at 4° C. Lysis were usually repeated, sometimes twice, using pre-warmed (37°C) Red Blood Cells Lysis Buffer. The cell pellet was washed twice by resuspension in PBS and recentrifugation for 2 minute at 4°C. Cells were enumerated using an automated cell counter. Cell

viability and differential cell counts were determined by trypan blue exclusion and Wright staining, respectively. Neutrophils purity was >95% and viability >90%. Cells concentration was adjusted with HBSS+ and maintained on ice until used in the various assays described below.

#### *Immunocytochemistry studies related to AGP binding to bovine neutrophils*

In order to verify if the activated bovine neutrophils were capable to bind AGP on the membrane surface,  $1.5 \times 10^5$  neutrophils were stimulated for 30 minutes with AGP at 0.3 mg/ml and 0.9 mg/ml.

The cells were centrifuged at  $500 \times g$  for 8 minute at  $4^\circ\text{C}$ , washed twice with cold PBS in order to remove unbound AGP molecules and stabilized with 1% formalin. Cells were allowed to settle on a glass slide and processed to assess AGP localization by immunofluorescence. After an incubation of 30 minutes at  $26^\circ\text{C}$  in PBS containing 1% BSA and 1% NDS (Normal Donkey Serum), the slides were incubated overnight at  $4^\circ\text{C}$  with a polyclonal rabbit anti-bovine AGP ( $17 \mu\text{g/ml}$  in Dulbecco-modified PBS) (Ceciliani 2007). After the incubation, the samples were washed twice in PBS and stained with a fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbit IgG antibody (1:200) for 30 minutes at  $26^\circ\text{C}$ . Chromatin DNA was stained with DAPI ( $0.05 \mu\text{g/ml}$  in PBS). As negative control, the primary antibody against AGP was omitted in one slide for each experiment. All samples were mounted with an anti-fade medium and observed with a conventional epifluorescence microscope (Nikon, Eclipse E 600).

#### *Neutrophil degranulation studies*

##### Experimental design for the stimulation of the cells.

Degranulation responses of neutrophils were investigated by studying the effect of AGP on the exocytosis of primary (azurophil) and secondary (specific) granules. All the experiments were carried out at  $37^\circ\text{C}$  in a sterile 96-well flat bottom ELISA plates (Becton Dickinson). Cells were incubated in different conditions as described below. The supernatant of each well was then transferred in an Eppendorf tube, centrifuged at  $400 \times g$  for 7 minute at room temperature (RT) and divided in two aliquots for the evaluation of granules exocytosis.

Primary and secondary granules release was determined by measuring the enzymatic activity of myeloperoxidase (MPO) (Quade 1997) and alkaline phosphatase (ALK-P) (Rausch 1975), respectively, which were assayed from the supernatants obtained from  $2 \times 10^6$  cells in 200 $\mu$ l. The assay of alkaline phosphatase enzymatic activity exocytosed by specific granules was carried out following a slight modification of a previously established protocol (coomber 1997). To 33 $\mu$ l of supernatant, 100 $\mu$ l of p-nitrophenyl phosphate (pNPP) ready made solution were added in 96 wells non sterile ELISA plates, and the mixture was incubated for 10 minute at RT. The reaction was finally blocked by the addition of 50 $\mu$ l NaOH 3M, and the plates were read at an absorbance of 410nm. MPO activity contained in primary granules was assayed on 50 $\mu$ l of supernatant: 200 $\mu$ l of tetramethylbenzidine (TMB) ready made solution were added. The reaction was carried out for 30 minutes at RT, and finally blocked by adding 50 $\mu$ l H<sub>2</sub>SO<sub>4</sub> 1M. The plates were read at an absorbance of 450 nm.

Background values were calculated from wells containing pNPP and TMB, in their respective assay, added with 33 $\mu$ l and 50 $\mu$ l of HBSS+ respectively, and the results were automatically subtracted from all values.

The data were expressed as the percentage of ALK-P, or MPO, activity compared to the total enzyme content of the cells, as determined after incubation of the same amount of cells with 0.5% hexadecyltrimethylammonium bromide (CTAB).

Supernatants were then transferred into 96-well flat bottom non sterile ELISA plates and enzymatic activities were measured on automatic microtiter plate reader Multiscan MS (Labsystem, Helsinki, Finland).

The experimental design was planned as follows:

Experiment 1. In a first series of experiments, the cells were incubated with the two different concentrations of purified AGP in order to study the effect of two different concentrations of AGP on spontaneous degranulation of cells. Negative controls were neutrophils incubated with an equal volume of HBSS+ instead of AGP. MPO and ALK-P activities were measured after 120 minutes of incubation at 37°C and at 5% CO<sub>2</sub> as previously described.

Experiment 2. In this second series of experiments, isolated neutrophils were incubated with two different concentrations of purified AGP and, in the meanwhile, challenged with ZAS. Positive controls were neutrophils incubated with ZAS and HBSS+ instead of AGP. Negative controls were non activated neutrophils in which equal volume of HBSS+ was added instead of ZAS and AGP.

In order to rule out any unspecific protein activation, cells were further challenged with 0.9mg/ml albumin. Time course incubation was carried out for 0, 15, 30, 60, 120 and 240 minutes.

Experiment 3 was designed in order to verify if AGP's activity was due to interaction of the protein with neutrophil membrane. Isolated neutrophils were therefore pre-incubated with two different concentrations of purified AGP for 10 minutes. Unbound protein was washed away by centrifugation (200 x g for 7 minutes) and cells were resuspended in HBSS+ and incubated with ZAS for 120 minutes. MPO and ALK-P activities were measured as described.

Experiment 4 was aimed to assess the importance of the glycan pattern of AGP for its degranulation-modulatory activity. In the last series of experiments de-sialylated AGP ( as-AGP) was prepared by treating purified AGP with 200 mU/ml neuraminidase (streptococcus 6646K, EC 3.2.1.51) in 0.01 M sodium phosphate buffer, pH 6.0, for 2h at 37°C, exactly as previously reported (Ceciliani 2007). Isolated neutrophils were then incubated with two different concentrations of purified as-AGP. In the last group of experiments, neutrophils exocytosis was also challenged with ZAS. Aliquots of protein which were not submitted to neuraminidase treatment were used as controls. ALK-P and MPO activities were measured after 120 minutes, following the protocols previously described.

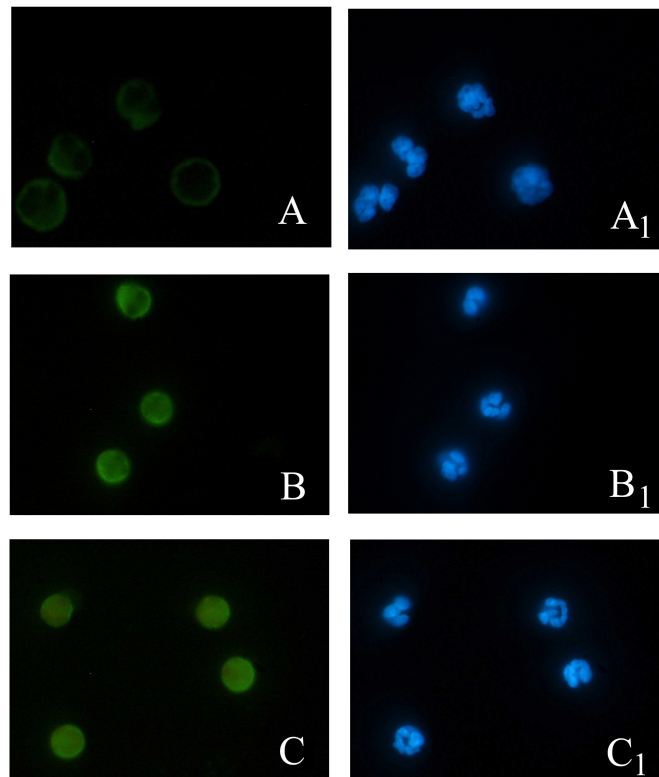
#### *Statistical analysis*

All statistical procedures were computed by using statistical software (SPSS 15.0, SPSS Inc., Chicago, USA). Results are expressed as mean values plus or minus standard error of the mean values. Different treatments were compared using a non parametric Wilcoxon test for paired samples. Statistical significance was accepted at  $P < 0.05$ .

## **Results**

### *AGP binds to the surface of neutrophils*

With the aim to investigate if AGP may react with the surface of cells, resting granulocytes were labelled for AGP immunoreactivity by using the polyclonal anti-bovine AGP antibody. Results are presented in Fig. 1. The incubation of the cells with AGP resulted in a homogeneous fluorescent staining of PMN surface. This anti-AGP immunoreactivity apparently increases when neutrophils are treated with acute phase concentrations of AGP (0.9 mg/ml) (Fig.1: panel C). Fig. 1 therefore shows that exogenous plasma AGP is capable to bind the surface of bovine neutrophils.



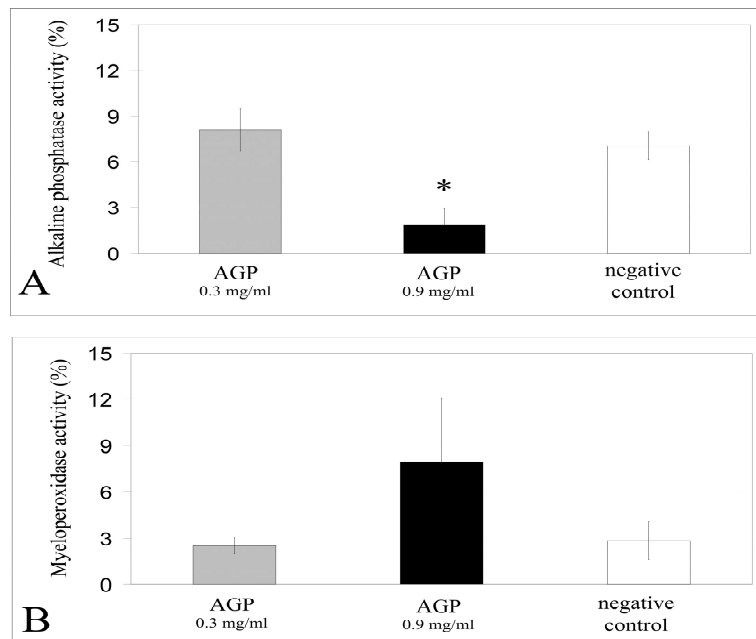
**Fig. 1: binding of AGP on the surface of neutrophil membrane**

Isolated neutrophils were treated with HBSS (Panel A), used as negative control, 0.3 mg/ml AGP (Panel B) and 0.9 mg/ml AGP (Panel C). Cells were immunostained with polyclonal rabbit anti-bovine AGP (Ceciliani et al., 2007) as primary antibody, and donkey anti-rabbit FITC conjugated secondary antibody. Right Panels (Panels A<sub>1</sub>, B<sub>1</sub> and C<sub>1</sub>) present the same cells after DAPI nucleus-specific staining (blue colour).



### *AGP modulates spontaneous degranulation (Experiment 1)*

The first series of experiments was designed to determine whether AGP may modulate spontaneous degranulation of secondary (specific) and primary (azurophilic) granules. Results are presented in Fig. 2. The treatment of cells for 120 minutes with AGP fulfils a protective effect against spontaneous exocytosis of secondary granules (Fig. 2A), since ALK-P activity, which was used as a marker of secondary granules exocytosis, dropped from  $7.04 \pm 0.92$  % (mean  $\pm$  SEM) of the HBSS treated cells to  $1.89 \pm 1.03$  % of AGP incubated cells ( $P < 0.05$ ). This effect is dose-dependent, the physiological concentration being non effective. The effect of AGP on primary granules spontaneous exocytosis is apparently opposite (Fig. 2B), since MPO activity, which was used as a marker of secondary granules exocytosis, increases from  $2.85 \pm 1.22$  % of the HBSS treated cells to  $7.97 \pm 4.01$  % of 0.9 mg/ml AGP-treated neutrophils. Due to the very high individual variability, this effect is not statistically significant. This effect of AGP on primary granules spontaneous degranulation can be appreciated only when cells are treated with acute phase concentration of AGP. Treating the cells with physiological concentration of AGP had apparently no effect.



**Fig. 2: the effects of AGP on neutrophil spontaneous degranulation** Isolated neutrophils (6 cows) were incubated with two different concentrations of AGP (0.3 mg/ml and 0.9 mg/ml) or HBSS (negative controls). After 120 minutes, alkaline phosphatase (ALK-P) (Panel A) and

myeloperoxidase (MPO) (Panel B) enzymatic activities were measured in the supernatant of neutrophil culture as markers for secondary and primary granules exocytosis, respectively. Values are expressed as percentages of ALK-P and MPO enzymatic activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean  $\pm$  SEM). Statistically significant differences between AGP and negative controls (neutrophils treated with HBSS instead of AGP) were indicated as \* ( $P < 0.05$ ).

*AGP modulates the exocytosis of secondary granules induced by ZAS (Experiment 2)*

The effects of AGP on secondary granules exocytosis are presented in Fig. 3A. The isolation procedure that was carried out to obtain the cells from clinically healthy animals was sufficient to induce a slight activation of neutrophils, as shown by the release of  $5.05 \pm 0.51$  % of the total ALK-P content from non stimulated cells at  $T_0$ . When neutrophils are stimulated with ZAS, which contains soluble C5a complement fraction derived from normal serum after activation with Zymosan, the enzymatic activity of ALK-P released from positive controls increases from  $12.92 \pm 2.95$  % ( $T_0$ ) to  $45.17 \pm 3.67$  % ( $T_{240}$ ).

The incubation of the cells with 0.9 mg/ml albumin, carried out to rule out an unspecific protein activation of the cells, gave no significant difference when compared to positive control, since it ranges from  $10.97 \pm 1.32$ % ( $T_0$ ) to  $42.57 \pm 6.44$ % ( $T_{240}$ ). When neutrophils are incubated with physiological concentration of AGP the response is bi-phasic and time-dependent: after the first 15 minutes lag it cannot be observed any statistically significant difference between AGP-treated cells, which ranges between  $10.88 \pm 2.59$ % ( $T_0$ ) and  $13.93 \pm 1.34$  % ( $T_{30}$ ), and positive controls, which ranges between  $12.92 \pm 2.95$ % ( $T_0$ ) and  $21.28 \pm 2.67$  % ( $T_{15}$ ). On the contrary, starting from  $T_{30}$ , it becomes evident that AGP, even when administered at physiological concentration, can reduce the activity of ALK-P. This inhibitory activity reaches its highest point at  $T_{240}$  ( $26.98 \pm 2.49$ % versus  $45.17 \pm 3.67$  % of positive controls).

The inhibitory activity of AGP toward secondary granules exocytosis is still more evident when neutrophils are treated with acute phase concentration of protein, similar to those found in plasma during the systemic response to inflammation. The degranulation of secondary granules is almost immediately inhibited when compared with positive controls (at  $T_0$  ZAS/AGP =  $6.71 \pm 1.87$ % versus positive

controls =  $12.93 \pm 2.95\%$ ). This inhibitory activity is steadily increasing over time, and reaches its highest point at  $T_{240}$  (ZAS/AGP =  $9.15 \pm 1.26\%$  versus  $45.17 \pm 3.67\%$  of positive controls). The down-regulating effect of acute phase concentration of AGP on secondary granules exocytosis is statistically significant starting from  $T_{15}$  incubation.

The effects of AGP on primary granules exocytosis are presented in Fig. 3B.

Again, the isolation procedure carried out to obtain the cells from clinically healthy animals was sufficient to induce a slight activation of neutrophils, as shown by the release of  $3.19 \pm 0.60\%$  (mean  $\pm$  SEM) of the total MPO content from non stimulated cells at  $T_0$ . The challenging of bovine neutrophils with ZAS induces an immediate exocytosis of primary granules, as indicated by the high MPO enzymatic activity at  $T_0$  ( $37.91 \pm 8.13\%$  of the total MPO activity). Incubation of ZAS-activated neutrophils with albumin (0.9 mg/ml) and AGP at both concentrations apparently has no effect on the primary granules exocytosis. Moreover, the release of MPO is not time-dependent, since it does not change from  $T_0$  to  $T_{240}$ .

In conclusion, the results presented in Fig. 3 demonstrated that the treatment of ZAS-activated neutrophils with AGP purified from bovine serum selectively reduces the release of secondary granule content in a dose-dependent way, and apparently has no effect on that of primary granules.

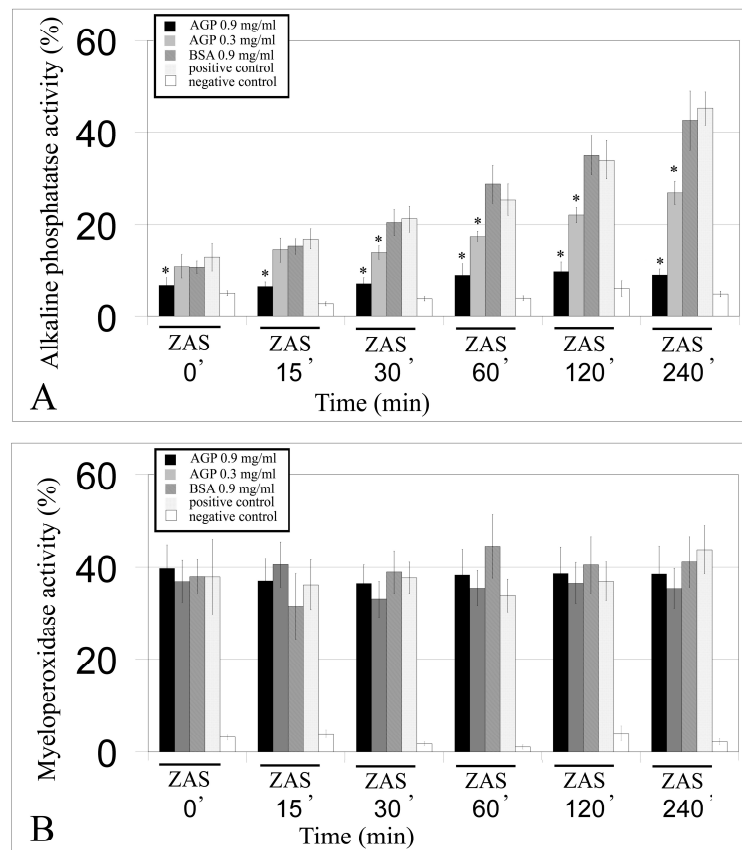


Fig. 3: **the effect of AGP treatment on secondary and primary granules exocytosis** Neutrophils were isolated from 7 cows and incubated with AGP at two different concentrations: physiological concentration (0.3 mg/ml) and acute phase concentration (0.9 mg/ml). Albumin (0.9 mg/ml) was used in order to rule out any unspecific protein activity. Degranulation of secondary granules was induced with ZAS as described in Materials and Methods. Positive controls were neutrophils treated with ZAS and HBSS instead of AGP. Negative controls were HBSS treated cells without ZAS. Panel A presents alkaline phosphatase (ALK-P) activity in the neutrophil culture supernatant, used as marker of secondary granules exocytosis. Panel B presents myeloperoxidase (MPO) activity in the neutrophil culture supernatant, used as marker of primary granules exocytosis. Values are expressed as percentages of ALK-P and MPO activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean  $\pm$  SEM). Statistically significant differences between the two concentrations of AGP and positive control (neutrophils activated with ZAS and treated with HBSS instead of AGP) were indicated as \* ( $P < 0.05$ ). The differences between positive and negative controls were all statistically significant, and were not indicated in the figure.

*The pre-incubation of cells with AGP reduces the exocytosis of neutrophil granules induced by ZAS (Experiment 3)*

In order to verify if one of the possible mechanisms of action of AGP was the interaction with the molecules that are believed to be involved in the binding of

ZAS-generated by-products, e.g. C5a receptor (i.e. CD88), isolated neutrophils were pre-incubated with AGP for 10 minutes, after which exocytosis was induced by ZAS. Results are presented in Fig. 4. The pre-incubation of cells with AGP does not modify the exocytosis of primary granules (Fig. 4B). On the contrary, Fig. 4A shows that the enzymatic activity of ALK-P used as marker for secondary granules exocytosis is decreased, from  $20.30 \pm 4.55\%$  of the positive controls to  $9.46 \pm 1.64\%$  of the AGP's (0.9 mg/ml) pre-incubated cells after 120 minutes of incubation with ZAS ( $P < 0.05$ ) While there is a decrease of secondary granules exocytosis also when cells are pre-treated with physiological concentration of AGP ( $14.64 \pm 2.18\%$ ), this result is not statistically significant when compared to positive control.

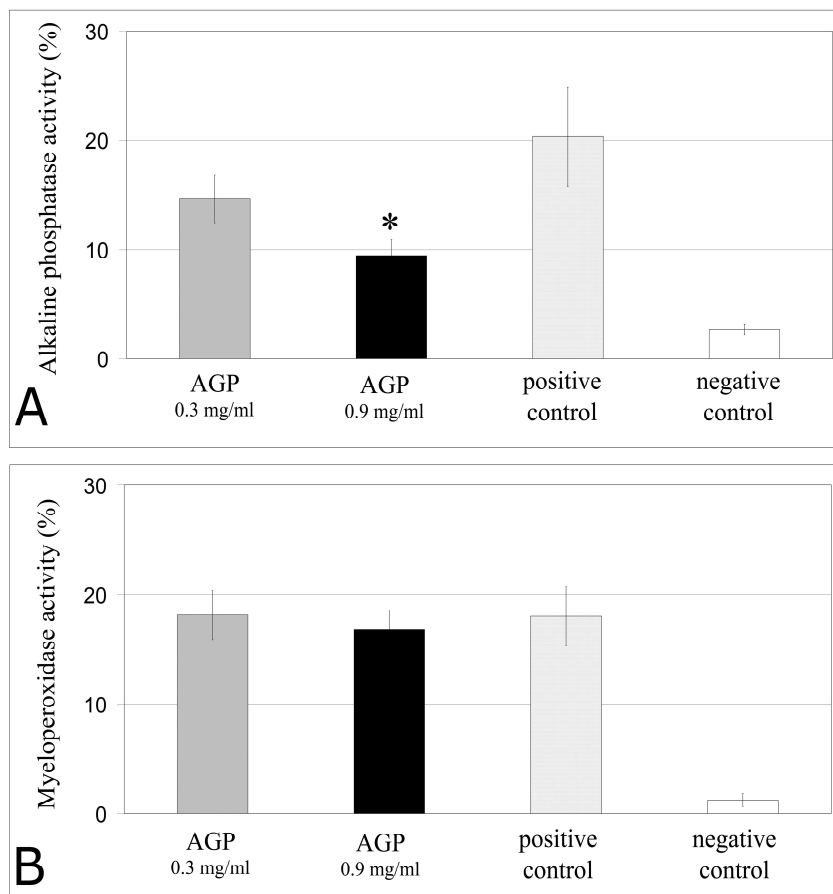


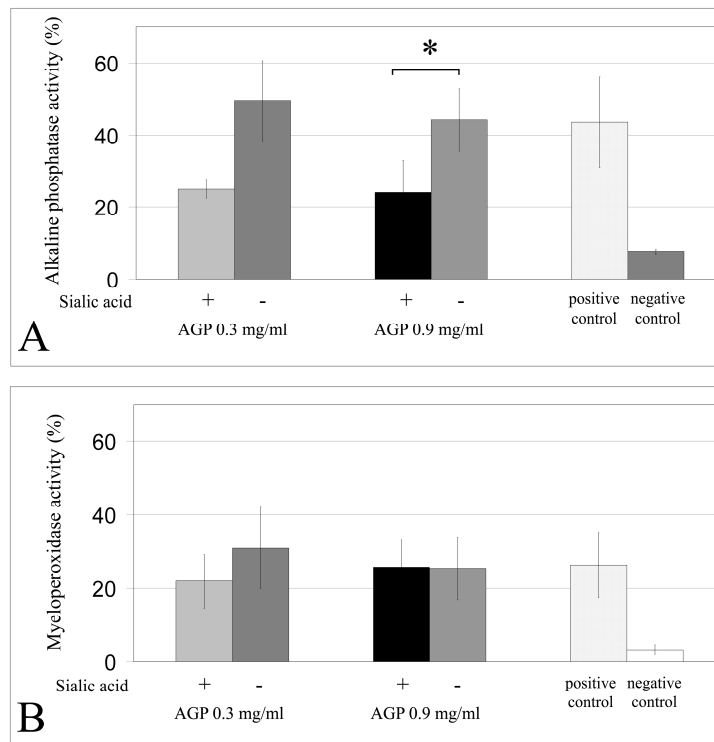
Fig. 4: **the effect of neutrophil pre-incubation with AGP on primary and secondary granules exocytosis** Neutrophils were isolated from 4 cows and pre-incubated for 10 minutes with physiological (0.3 mg/ml) and acute phase (0.9 mg/ml) concentrations of AGP, and HBSS. Unbound protein was removed by centrifugation, and degranulation was induced by treating the

cells with ZAS for 120 minutes. Negative controls were HBSS treated cells without ZAS. Positive controls were neutrophils treated with ZAS and pre-incubated with HBSS instead of AGP. Alkaline phosphatase (ALK-P) (Panel A) and myeloperoxidase (MPO) (Panel B) enzymatic activities in the neutrophil culture supernatant were used as marker of secondary and primary granules exocytosis, respectively. Values are expressed as percentages of ALK-P and MPO enzymatic activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean  $\pm$  SEM). Statistically significant differences between the two concentrations of AGP and positive control were indicated \* (  $P < 0.05$ ). The differences between positive and negative controls were all statistically significant, and were not indicated in the figure.

*AGP effects on degranulation are related to its sialic acid content (Experiment 4).*

The last set of experiments was set out to determine the importance of the sialic acid residues exposed on the surface of AGP. Exocytosis studies were then performed using in parallel AGP aliquots purified from bovine serum and neuraminidase-treated AGP (as-AGP), in which terminal sialic acid residues were enzymatically removed. The reaction was carried out for 120 minutes. When neutrophils were activated with ZAS, as-AGP lost every inhibitory activity, which is retained by the olo-protein (Fig. 5A). The desialylation of AGP has no effect on primary granules (Fig. 5B). The spontaneous exocytosis down-regulatory effects of AGP on secondary granules is equally lost when sialic acid terminal residues are removed, as shown in Fig. 6A as well as the apparently up-regulatory activity on primary granules exocytosis, as shown in Fig. 6B.

In conclusion, both inhibitory and up-regulatory effects on secondary and primary granules exocytosis, respectively, cannot be detected by using the desialylated protein.



**Fig. 5: the effects of desialylation of AGP on its modulatory activity of neutrophil exocytosis.** Isolated neutrophils (7 cows) were incubated with physiological (0.3 mg/ml) and acute phase (0.9 mg/ml) concentrations of AGP before and after treatment with neuraminidase, which specifically removes terminal sialic acid residues. Degranulation of secondary (Panel A) and primary (Panel B) granules was induced with ZAS as described in Materials and Methods. HBSS and ZAS alone, without adding AGP, were used as positive control. Negative controls were HBSS treated cells without ZAS. After 120 minutes, alkaline phosphatase (ALK-P) (Panel A) and myeloperoxidase (MPO) (Panel B) enzymatic activities were measured in the supernatant of neutrophil culture as markers for secondary and primary granules exocytosis, respectively. Values are expressed as percentages of ALK-P and MPO enzymatic activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean  $\pm$  SEM). Statistically significant differences between the two indications of AGP and HBSS treated activated neutrophils (positive control) indicated as \* ( $P < 0.05$ ). The differences between positive and negative controls were all statistically significant, and were not indicated in the figure.

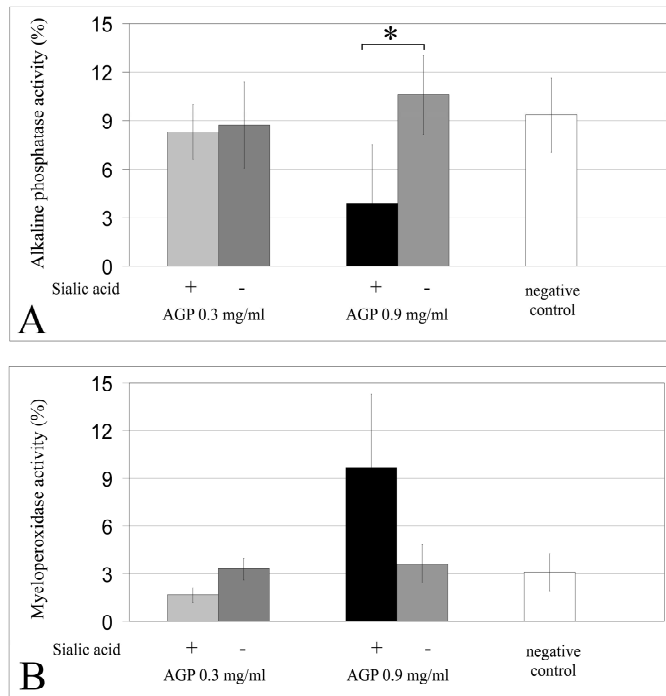


Fig. 6: **the effects of desialylated AGP on neutrophil spontaneous degranulation.** Isolated neutrophils (5 cows) were incubated with two different concentration of AGP (0.3 mg/ml and 0.9 mg/ml) before and after treatment with neuraminidase, which specifically removes terminal sialic acid residues. HBSS instead of AGP was used as negative controls. After 120 minutes, alkaline phosphatase (ALK-P) (Panel A) and myeloperoxidase (MPO) (Panel B) enzymatic activities were measured in the supernatant of neutrophil culture as markers for secondary and primary granules exocytosis, respectively. Values are expressed as percentages of ALK-P and MPO enzymatic activity compared to total ALK-P and MPO activity in neutrophils lysed with CTAB (mean  $\pm$  SEM). Statistically significant differences between AGP and HBSS treated neutrophils (negative control) were indicated as \* ( $P < 0.05$ ).

## **Discussion and Conclusions**

The activity of neutrophils is essential for the development of the first phase of innate immunity. Yet, they are very aggressive cells, and the extracellular release of ROS products, as well as the proteolytic enzymes contained in their granules, may cause massive tissue injuries during acute and chronic inflammation. Many acute phase proteins fulfil anti-inflammatory functions (Gabay 1999) directed toward the inhibition of these collateral damages of inflammation. In the first part of this study we provide evidence that one of them, the minor acute phase protein  $\alpha$ 1-acid glycoprotein, can modulate one of the most important functions of bovine



neutrophils, the mobilization of their granules. Spontaneous degranulation is affected in a dose-dependent way by AGP. The biological significance of this result is unknown, but we may speculate that at least one of the functions of the rise in serum concentration of AGP during acute phase reaction might be that of counteract a praecox degranulation of neutrophils while they are still in the blood stream. The apparently opposite effect on primary granules has still to be elucidated. The capability of AGP to down-regulate the mobilization of secondary granules was more evident when the cells were challenged with ZAS.

The pathways regulating the specific mobilization of neutrophil granules are far to be clarified; nonetheless, granules translocation is known to depend on highly specific signals, i.e. their mobilization shows to be hierarchical, since primary granules are translocated after the secondary ones (Tapper 1996). Still it is to be queried how AGP might interfere with the molecular pathways driving to the exocytosis of neutrophil granules. AGP binds on the surface of neutrophil membrane, as shown by immunocytochemistry studies. It is therefore at least conceivable that the activity of AGP is due to the interaction with the protein on the surface of the cells. In order to verify the functional significance of this interaction, neutrophils were pre-treated with AGP, and then activated with ZAS. Since priming of neutrophils with AGP reduces the secondary granules exocytosis, it can be hypothesized that AGP might, at least partially, act by competing with the specific pathways which are activated by the pro-inflammatory challengers used to stimulate the cells. Essentially the treatment of serum with Zymosan generated C5a that activated the cells by interacting with CD88, its specific receptor. It would be very interesting to determine whether the reduced exocytosis induced by AGP is due to the interaction of the protein with CD88, which may contribute to down-regulate the secondary signals necessary to integrate the inflammatory response triggered by C5a.

And through still undisclosed mechanisms, AGP might modulate neutrophil granule exocytosis by binding to its specific receptors exposed on the surface of the cells. AGP's binding sites on neutrophils are still poorly understood: at least two different binding sites have been identified on human granulocytes surface, with different binding affinities (Shiyan 1997). The molecular pathways activated

when these receptors interact with AGP are still unknown. AGP's receptors on the surface of neutrophils are likely involved in cytoskeletal remodelling events that drive to the selective mobilization of granules. This hypothesis is supported by a recent finding (Gunnarsson 2007) which reported that AGP triggers  $\text{Ca}^{2+}$  mobilization in human neutrophils. Since the hierarchical mobilization of granules is due to different cytoskeletal activation, which is calcium dependent, we may speculate that the effects of AGP on the modulation of secondary granules traslocation are related to its capability of inducing the intracellular  $\text{Ca}^{2+}$  mobilization.

Another possible mechanism of action of AGP can be assumed. As a binding protein, AGP might also act by sequestering some of the mediators, thus reducing their biological availability in the inflammatory environment. Further experiments should support such hypothesis; anyway, AGP can bind some inflammatory mediators such as PAF (Ojala 2006), as already shown in humans.

All the exocytosis modulating activities of AGP shown in this paper are strongly related with its glycan moiety, since all of them were suppressed by the removal of sialic acid terminal residues exposed on the protein surface. These results are consistent with other reports (Costello 1979, Ceciliani 2007) demonstrating that the sialic acid residues are essential to some functions of the protein. A remarkable knowledge, since the glycosylation of AGP is strongly dependent on its physiological and pathological status. Furthermore, in accordance with above findings, other authors (Gunnarsson 2007) had previously demonstrated that sialic acid molecules exposed on the surface of the AGP are essential to increase  $\text{Ca}^{2+}$  concentration. Comparing the effect on granule exocytosis of plasma AGP and the hypersialylated and hyperfucosylated glycoforms present in secondary granules of neutrophils (Theilgaard-Monch 2005, Rahman 2008), or in serum during several inflammatory diseases (Ceciliani 2007) would be an interesting matter of investigation. In conclusion, our findings reported for the first time that AGP is able to specifically down-regulate the secondary granule exocytosis. Signalling mechanisms underlying the involvement of AGP in modulating neutrophil functions are still not known, and further studies are necessary to go insight the signalling capability of this immunomodulatory protein.

**AGP EFFECTS IN HUMAN ENDOTHELIAL CELLS**  
**AND PMN ADHESION**

## **Introduction**

Angiogenesis is an important host response to injury, ischemia, and is crucial to the wound healing process. Angiogenesis is also central to tumor cell survival. As a tumor mass grows, it must promote new vessel formation to maintain a continuous supply of nutrients of oxygen, some tumors generate multiple proangiogenic factors and signaling to encourage tumor angiogenesis.

Angiogenesis or neovascularization is the process of generating new blood vessels derived from existing vascular structures. It is a multistep process that involves endothelial cells (Auerbach 2003). First, the basement membrane within the wall of a preexisting vessel is proteolytically degraded allowing ECs to disengage from neighboring cells and to migrate through the vessel wall to migrate towards the angiogenic stimulus (Auerbach 2003, Karamysheva 2007). Proliferation occurs behind the migrating front, to deliver an adequate number of cells to form the new vessel. The last step is the reorganization of the new migrated cells into a three dimensional tubular structure. In adults, the 4 steps of new vessel formation, 1) basement membrane disruption, 2) cell migration, 3) cell proliferation, and 4) tube formation (Auerbach 2003), are tightly regulated, and only activated under very defined conditions like wound healing (Karmaysheva 2007).

The processes of cell adhesion, migration, invasion and proliferation are mediated, in part, by cell adhesion molecules (Brooks 1996) expressed by the endothelium. Some of these adhesion molecules include integrins like  $\alpha v \beta 3$  which is upregulated during angiogenesis (Brooks 1994), immunoglobulins like VCAM-1 (Koch 1995), and selectins like E-selectin, which is surface expressed on cytokine-activated vascular endothelium. Moreover, specific antibodies targeting E-selectin have been shown to block capillary tube formation, *in vitro* (Nguyen 1993).

Bovine serum AGP binds to bovine pulmonary microvascular endothelial cells (Schnitzer 1992) in a calcium independent manner that does not utilize lectin-like receptors, such as the asialoglycoprotein or mannose receptors. Endothelium

binding sites specific for AGP, have been demonstrated but not fully characterized (Predescu 1998). AGP binding studies in a bovine system revealed comparable results as that seen in a human system. By binding to the endothelium, AGP plays a vital role in maintaining capillary permselectivity. It has been shown to be of crucial importance in maintaining the capillary charge barrier in rat skeletal muscle (Haraldsson 1987), guinea-pig skin (Muchitsch 1996), frog mesenteric capillaries (Curry 1989) and rat glomerular capillaries (Haraldsson 1992). Sorensson et al. also found that human dermal microvascular endothelial cells produced AGP that was immunolocalized to the glycocalyx where it helped to maintain the capillary barrier for macromolecules (Sorensson 1999). Transcytosis of AGP across the microvascular endothelium occurs rapidly, exclusively via caveolae (Predescu 1998). After its perfusion AGP can be detected in the perivascular space within one. As anticipated, AGP was not detected within intercellular space.

Under physiologic conditions, the endothelium is not adherent for peripheral blood cells. Upon stimulation, the endothelium surface expresses multiple adhesion molecules, including P-selectin, E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Hack 2001). During injury and inflammation, the endothelial surface becomes adherent for circulating leukocytes that adhere to, migrate across and traverse between the endothelial cells to enter the site of inflammation (Dauphinee 2005). Neutrophil adherence to vascular endothelial cells is the initial event in the emigration of neutrophils through blood vessels walls to tissue site of inflammation (Tonnesen 1984). The first contact between neutrophils and endothelium of postcapillary venules is known as capture or tethering and is mediated by P and E-selectins expressed on the endothelium that bind to both P-selectin glycoprotein ligand 1 (PSGL1) and sialylated carbohydrates present on the surface of the neutrophils (Ley 2007, Zarbock 2008). This first PMN to- endothelium contact will cause the neutrophils to slow down and to start rolling along the activated endothelium surface, integrating different chemokines that will cause the activation of the

neutrophil and will slow the rolling. The last step of the neutrophil recruitment is mediated by the integrins inter-cellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), that will cause the neutrophil to stop and finally to crawl and transmigrate across the endothelium through the paracellular junctions to get to the inflammation site.

Neutrophils constitute the first line of defense against invading bacteria (Zarbock 2008). Neutrophil activation and recruitment to the inflammation site is not fully understood, but seems to be gradual and mediated by several signaling pathways (Ley 2002). While essential to oppose infections, the activation of neutrophils can also release cytotoxic mediators, which may result in tissue damage. Therefore, neutrophil recruitment during inflammation is paradoxical; they are crucial for host defense, but if their recruitment and activation becomes dysregulated, they may also be involved in the development of tissue injury and organ failure (Rahman 2008).

Taken together, the data clearly shows that endothelial cells produce, bind, and transport via a caveolae-dependent pathway, AGP. Further, AGP inhibits the action of histamine on endothelial cells in a cAMP-dependent manner (Sorensson 2000). In the current studies, we studied the effect of AGP on *in vitro* angiogenesis, and PMN adhesion to endothelia.

Plus since AGP, due to its glycan moiety, has a high affinity to E-selectin on the surface of endothelial cells (Cecilian 2007) and E-selectin is known to be overexpressed in cytokine stimulated endothelium we wondered if AGP, as an anti-inflammatory molecule would have any effects on the PMN adhesion to a monolayer of HVEC-Ls.

## **Materials and Methods**

**Preparation of Human  $\alpha_1$ -acid Glycoprotein:** AGP was isolated by conventional HPLC ion exchange chromatography as described (Ceciliani 2007) with slight modifications. Briefly, human serum was dialyzed against the buffer used for the initial chromatography (10 mM citrate–phosphate buffer, pH 4.0). The serum was centrifuged to remove proteins not soluble at pH 4.0, and the supernatant was applied to an HiTrap Q Sepharose strong anionic exchange column 1 ml (GE Healthcare) equilibrated with the starting buffer. The protein was eluted in 100 mM citrate–phosphate buffer, pH 4.0. The fraction containing AGP was concentrated with Centricon 10 (Millipore) to 1/10 of initial volume and directly loaded onto an HiTrap SP Sepharose (GE Healthcare) strong cationic exchange column, equilibrated in 100 mM citrate–phosphate buffer, pH 4.0. A final purification step was performed to remove endotoxins potentially introduced during the purification procedure. The fraction containing AGP was directly loaded on a Sephasil Protein C4 (5  $\mu$ m ST 4.6/100 mm) chromatographic column (GE Healthcare) equilibrated with 0.065% TFA in 2% acetonitrile. Protein separation was accomplished using a 0–65% gradient of acetonitrile + 0.05% TFA over 33 min, at a flow rate of 1 mL/min. The fraction containing AGP was collected, concentrated to 1 mg/mL in a Savant microconcentrator, aliquoted and stored at -80°C.

**Endothelial Cell Culture:** Human lung microvascular endothelial cell (HMVEC-L)s (Lonza) were cultured in EC growth medium (EBM-2, Clonetics) containing 5% fetal bovine serum (Hyclone Laboratories, Logan, UT), human recombinant epidermal growth factor (EGF), human recombinant insulin-like growth-factor-1, human basic fibroblast growth factor, vascular endothelial growth factor, hydrocortisone, ascorbic acid, gentamicin, and amphotericin as described (Gong 2008). Only HMVEC-Ls in passages 6 to 8 were studied.

**EC Tube Formation Assay:** Each well of a 96-well plate was coated with 30  $\mu$ l of Matrigel (10 mg/ml; BD Biosciences, Bedford, MA) using a modification of previously described protocol (Yang 1996). Briefly, 30  $\mu$ l of Matrigel on ice were allowed to polymerize for 1h at room temperature in each of the wells, followed by 1h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. HMVEC-Ls were seeded at  $2 \times 10^4$  cells/well onto the Matrigel-coated wells and cultured for 6h in medium, in the presence or absence of increasing concentrations of AGP, BSA or media alone and observed for tube formation. At 6h, tubular structures were photographed and quantified with a Nikon inverted microscope through a 20X objective.

**EC viability assay:** HMVEC-L's at  $10^4$  cells per well were seeded into the wells of a 96-well plate for 24h at 37°C. The monolayers were treated for 0.5h or 6h with AGP 0.75mg/ml or medium alone. Cells were then washed with serum-free medium, incubated for 4h at 37°C with 0.5mg/ml of MTT (Sigma) followed by incubation with 10%SDS+0.01N HCl to solubilize the formazan product. Plates were read at A<sub>540nm</sub> (Versamax microplate reader, Molecular devices, Sunnyvale CA).

**EC Adhesion Assay:** HMVEC-Ls were seeded in matrigel-coated wells of a 96 well plate at  $3 \times 10^4$  cells/well and incubated for 30 min at 37°C in 0.1%BSA/DMEM, in the presence or absence of increasing concentrations of AGP. After washing x2, adherent cells were stained with 0.2% crystal violet in 20% methanol, washed with PBS, and the residual stain extracted with 1% SDS, and the A<sub>560nm</sub> of extracts measured.

**Migration in a Wounding Assay:** HMVEC-Ls were seeded at  $2 \times 10^5$  cells/well in the wells of a 24 well plate (Corning Glass, Corning, NY) and cultured to confluence. Using a sterile 200 $\mu$ l pipette tip, a single wound was made across the diameter of each monolayer, after which cell debris was removed by washing with HEPES as described (Liu 2009). The wounded monolayers were then incubated for 24h with AGP 0.75mg/ml or media alone. Photomicrographies of each well



were taken at times 0h, 5h, and 24h with a Nikon inverted microscope through a 4X objective. In selected wash-out experiments, after 24h, monolayers were incubated with media alone and two more photomicrographies were taken at time 36h and 48h.

**Endothelial Cell Chemotaxis Assay:** Gelatin-impregnated polycarbonate filters (13 mm diameter, 8.0  $\mu$ M pore size; Nucleopore Inc., Pleasanton, CA) were mounted in chemotactic chambers (Neuroprobe Inc., Gaithersburg, MD) as described (Sun 2001). HMVEC-Ls ( $5 \times 10^4$  cells in 100  $\mu$ l) were added to the upper compartment of each assay chamber. Each lower compartment contained 0.5% FBS as the chemoattractant. In selected experiments, AGP was introduced into either the upper or lower compartment in the presence or absence of FBS. After a 5h incubation (37°C, 5% CO<sub>2</sub>), filters were removed, fixed (3.7% formaldehyde in H<sub>2</sub>O, 30 min), washed, stained with 0.5% crystal violet in 25% ethanol, washed, and the top surface of each filter scraped free of cells. The crystal violet was then extracted from each filter with 0.1M citric acid in 50% ethanol for 30 min and the A<sub>560nm</sub> of extracts measured.

**Preparation and Fluorescent Labeling of PMNs:** Whole peripheral blood from healthy human volunteers was collected under a protocol approved by the University of Maryland, Baltimore, Institutional Review Board, into acid citrate dextran (Sigma) solution, and PMNs were isolated by dextran erythrocyte sedimentation and density gradient centrifugation through ficoll-hypaque (Sigma) as previously described (Sakarya 2004). PMNs were resuspended in HBSS without divalent cations (HBSS<sup>-</sup>) at  $5 \times 10^6$  PMNs/ml and were incubated with 5  $\mu$ M CalceinAM (Molecular Probes, Eugene, OR) for 30 min at 37°C (Welten 2004). PMNs were washed three times with HBSS<sup>-</sup> after which their purity was >95% and viability >98% by trypan blue dye exclusion.

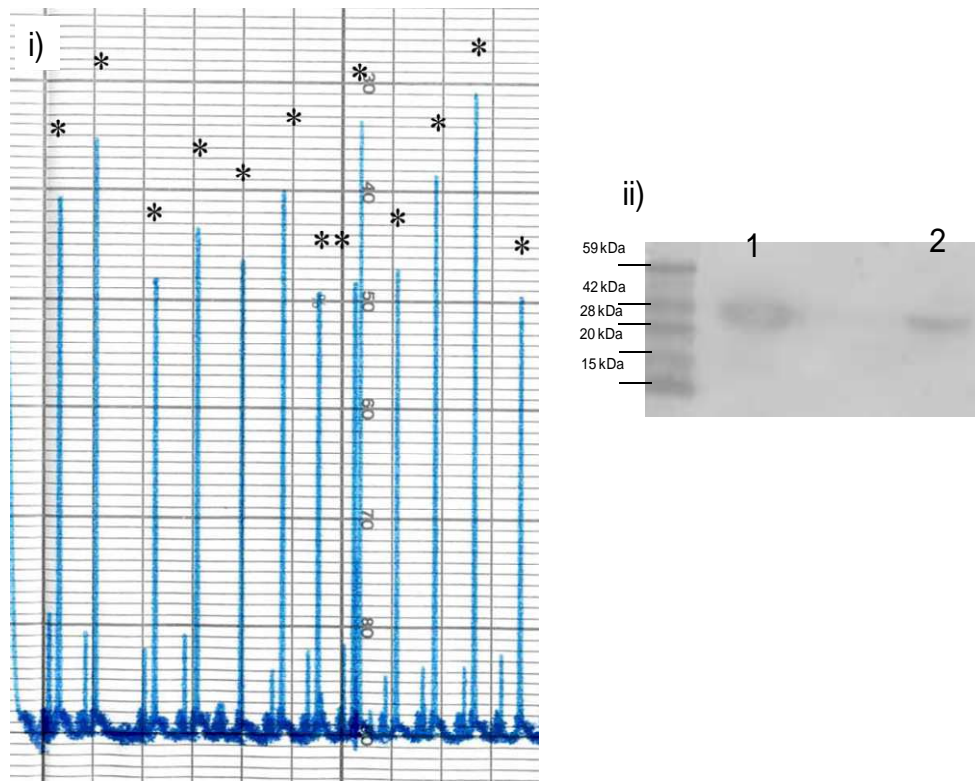
**PMN Adhesion Assay:** HMVEC-Ls were seeded ( $1 \times 10^4$  cells/well) into the wells of 96-well culture plates (Costar, Cambridge, MA), and cultured to confluence (48 h, 37°C, 5% CO<sub>2</sub>). The HMVEC-L monolayers were preincubated

for 5h with LPS 100 ng/ml or media alone. CalceinAM-labeled PMNs were pretreated for 15 min with fMLP ( $10^{-6}$  M)/cytochalasin B (5  $\mu$ g/ml) or media alone, washed, and incubated for 30 min at 37°C with HMVEC-L monolayers ( $5 \times 10^4$  PMNs/well) in the presence or absence of increasing concentrations of AGP or media alone. After gentle washing to remove nonadherent PMNs, the attached PMNs were fluourometrically assayed (excitation 485 nm, emission 538nm) in a Cytofluor II Multi-well Fluorescence plate reader (PerSeptive Biosystems, Framingham, MA). For each experiment,  $5 \times 10^4$  labeled PMNs were used as a standard to which fluorescence units of adherent PMNs adhesion were compared and expressed as % adhesion ( adherent PMNs / total PMNs x 100%).

**Statistical Methods:** Student t-test, with a two tailed distribution and an homoscedastic variance was used for all statistical analyses (Microsoft Office Excel 2003). Significance accepted for p values <0.05.

## Results

**Preparation of Human  $\alpha_1$ -acid Glycoprotein:** AGP was isolated from human serum by conventional HPLC ion exchange chromatography, using three different columns and finally getting a purified, endotoxin free, single protein.



**Figure 1. Preparation of Human  $\alpha_1$ -acid Glycoprotein:** On the left (i) chromatogram of HiTrap SP Sepharose column, where AGP is eluted in the void volum. \* indicate de absorbance pics, containing AGP that were collected and later analized. On the right (ii) Coomassie staining of the electrophoretic gel containing the purified protein. Lane 1 was loaded with 5 $\mu$ g of protein whereas lane 2 was loaded with 1 $\mu$ g of total protein. We can see a single band, showing that the final solution contains a single protein, and it is about 42kDa which is the expected molecular weight.

**AGP Inhibits Endothelial Cell Capillary-Like Tube Formation:** We asked whether the heavily sialylated, acute phase protein, AGP, might influence EC tube formation, *ie in vitro* angiogenesis, in a two-dimensional Matrigel system. After 6h in the absence of AGP, a mean ( $\pm$ SE) of  $32.5 \pm 3.4$  tubes/HPF were detected (Figure 2A and B). After 6h in the presence of increasing concentrations of AGP, it was possible to see an inhibition of the tube formation at concentrations as low as 0.25mg/ml even if they were not enough to be statistically significant. AGP at  $\geq 0.50$ mg/ml inhibited tube formation  $>70\%$  with a maximum effect of 97% inhibition at 0.75mg/ml (Figure 2B). The statistical significance started at 0.50mg/ml with a 73.8% decrease on the tube formatting and it reached it's highest level at 0.75mg/ml with a 97.4% of inhibition. Showing that AGP inhibits EC tube formation on matrigel in a dose dependent manner. Bovine Serum Albumin (BSA) (Sigma) was chosen as a simultaneous protein control, and identical experiments were performed with the identical increasing concentrations of BSA but no significant activity could be seen (data not shown).

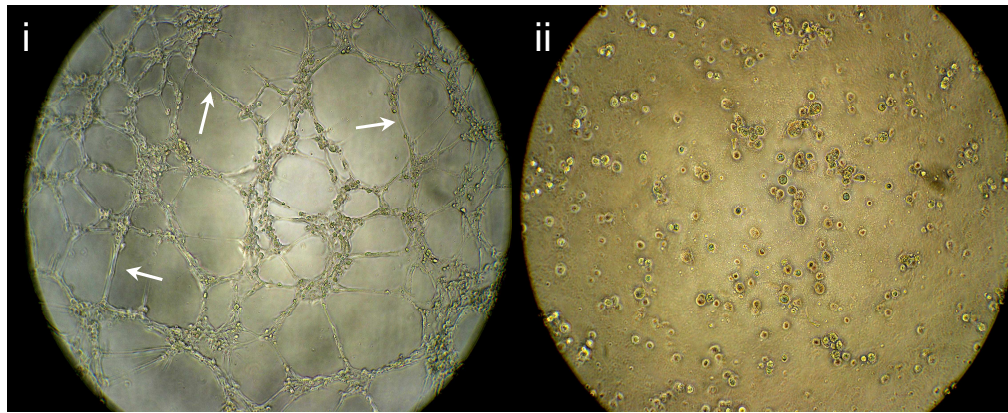
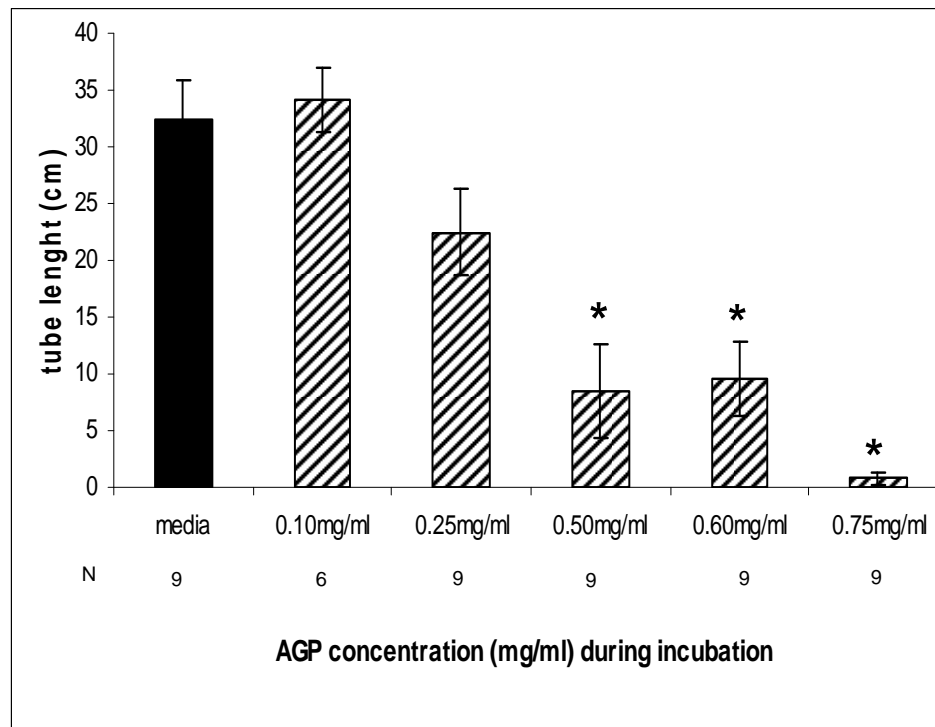


Figure 2A. (i) On the left, HMVEC-Ls were incubated for 6h on Matrigel. Arrows indicate capillary-like tubes. (ii) Identical conditions as described in (i) except the HMVEC-Ls were incubated in the presence of 0.75mg/ml AGP. Magnification 4X.



**Figure2B. AGP Inhibits Endothelial Cell Capillary-Like Tube Formation:** HMVEC-Ls were treated with increasing AGP concentrations, from 0mg/ml to 0.75mg/ml, and incubated for 6h on Matrigel in the wells of 96 wells plates. Photomicrographies were taken and tube length measured. Each bar represents mean (+/- SE) tube length, \* indicates significantly decreased compared to the simultaneous medium control at  $p < 0.05$ .

**EC viability assay:** A MTT conversion assay was introduced to exclude AGP-induced EC cytotoxicity; after incubation of EC for 6h with 0.75mg/ml AGP, no loss of cell viability could be detected (data not shown). Therefore, AGP at 0.75mg/ml does not provoke HMVEC-L cytotoxicity within 6h.

**AGP Inhibits EC Adhesion to Matrigel:** We now have established that AGP inhibits the multistep process of *in vitro* angiogenesis (Figure 2). We asked whether this negatively-charged molecule might exert its anti-angiogenic effect through altered EC adhesion to the Matrigel substrate. Incubation of ECs with increasing concentrations of AGP promoted a biphasic effect of EC adhesion to Matrigel (Figure 3). At lower concentrations of 0.05-0.25mg/ml, AGP increased

EC adhesion with a maximal effect of +24.5% at 0.10mg/ml (Figure 3). In contrast, AGP at concentrations of  $\geq 0.50$ mg/ml decreased EC adhesion with mean maximal inhibition of -29.7% at 0.75mg/ml (Figure 3). These combined data indicate a biphasic AGP activity, at lower concentrations, AGP increased EC adhesion to Matrigel, whereas at higher concentrations, it diminished it.

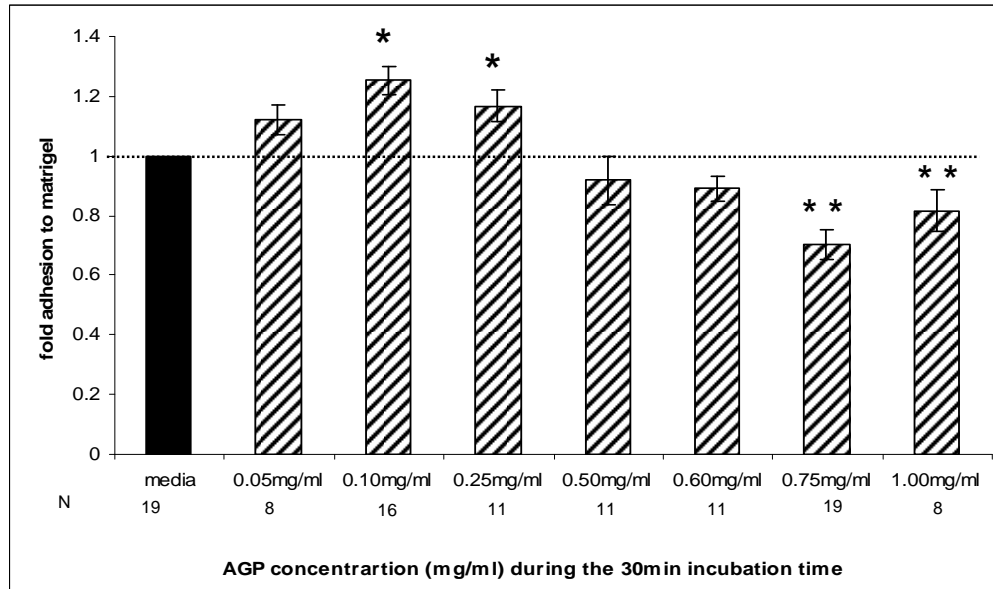
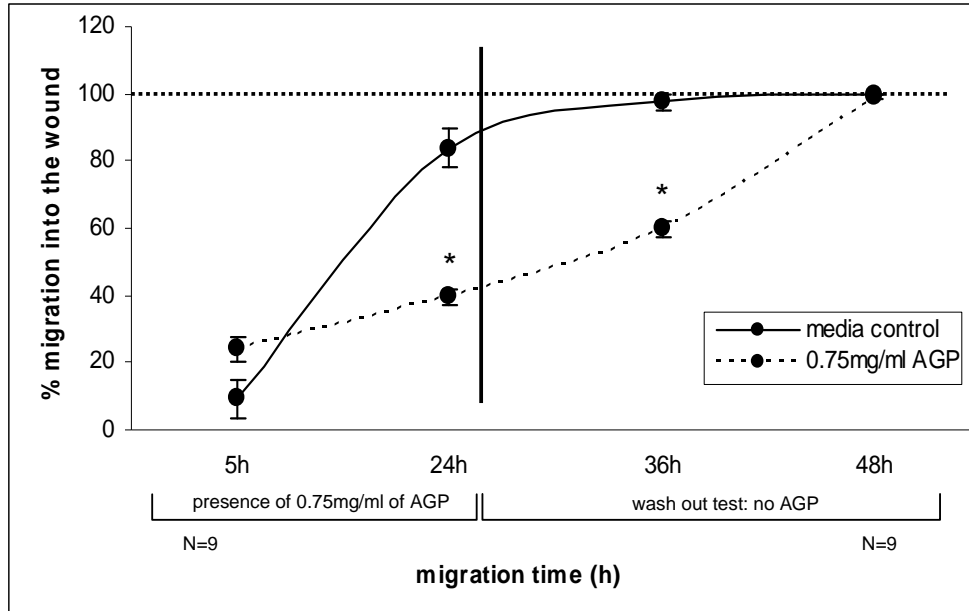


Figure3. **AGP Inhibits EC Adhesion to Matrigel:** HMVEC-Ls at  $3 \times 10^4$  cells per well were incubated for 30min in matrigel coated wells in the presence of increasing concentrations of AGP. After non adherent cells were washed away, remaining adherent cells were counted with a colorimetric assay (crystal violet dye) and then expressed as mean ( $\pm$  SE) normalized to adhesion of the simultaneous medium control (closed bar). \* indicates significantly increased compared to the medium control at  $p < 0.05$ . \*\* indicates significantly decreased compared to the medium control at  $p < 0.05$ .

**AGP Inhibits EC Migration in a Wounding Assay:** We already know that AGP influences EC tube formation on (Figure 2) and EC adhesion to the glycocalyx (Matrigel) (Figure 3). We wondered if AGP could also inhibit EC migration into a wound. In a wounded HMVEC-L monolayer, we observed wound healing over time (cells migrated into the wound) in presence or absence of 0.75mg/ml of AGP. In the absence of AGP, the wound was 84% reconstituted by 24h, whereas in presence of AGP there was only 40% reconstitution. Therefore, AGP inhibited wound healing by 44%. At 24h, a wash out experiment was performed in which AGP was removed and cells were allowed to continue to heal. The wound reached

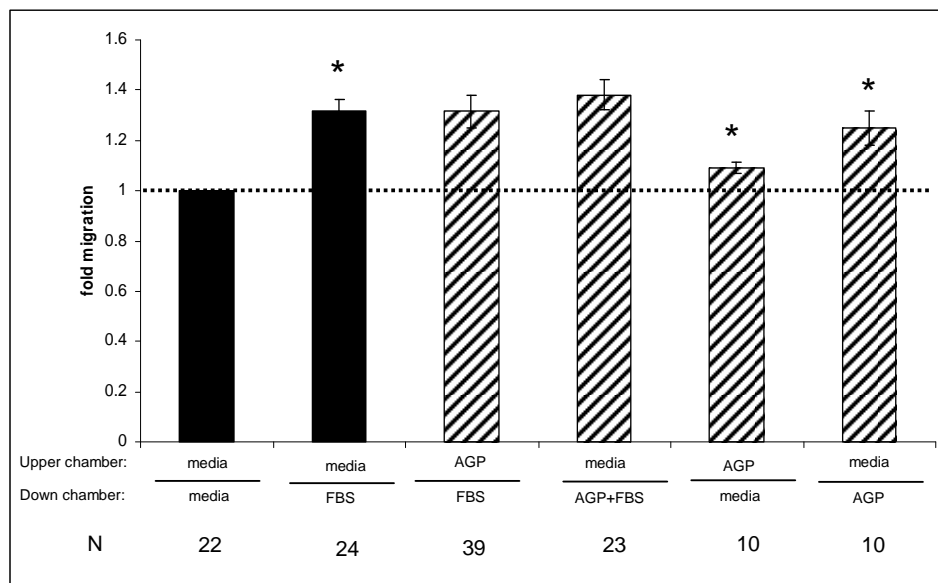
a 60% healing 12h after the AGP was removed and totally healed by 24h. These data indicate the inhibitory effect of AGP at 0.75mg/ml over 24h was reversible and excludes irreversible loss of viability.



**Figure4. AGP Inhibits EC Migration in a Wounding Assay:** Wounded EC monolayers were cultured for 24h in the presence of AGP 0.75mg/ml or media alone and photomicrographies were obtained at 0h, 5h and 24h. At 24h, wash-out experiments were performed in which all wounded monolayers were cultured in medium alone and photomicrographes were again obtained at 36h and 48h. t0h was taken as 0% migration for each wound. %migration mean (+/- SE) is shown. \* indicates significantly decreased ( $p < 0.05$ ) migration compared to the simultaneous media controls. Amount of migration was quantified from the photomicrographes using GIMP (GIMP development).

**AGP Influences EC Motility:** Since AGP influences EC adhesion to the underlying ECM (matrigel) (Figure 3), EC tube formation (Figure 2) and migration in the wound healing assay (Figure 4), we asked whether this same protein might also regulate EC chemotaxis. In a modified Boyden chamber, AGP was introduced into either the upper or lower compartments, in the presence or absence of a chemotactic gradient provided by FBS in the lower compartment, and EC migration through the pores in the membrane filter assayed (Figure 5). When FBS was placed in the lower compartment we obtained a +32% migration, since FBS is known to be a chemoattractant. Since AGP is known to bind and

neutralize multiple endogenous mediators (Israili 2001), we tested FBS chemotactic activity in the presence and absence of AGP and we couldn't see any influence of AGP in the migration behavior when FBS was present whether we tested AGP as a chemoattractant or a chemokinetic. Indicating thus that AGP if AGP binds LPS it does not dampen its chemotactic activity against EC. When AGP was tested alone, without the presence of FBS, it increased EC chemotaxis in both cases, by an 8% when it was used on the top chamber, and by a 25% when it was used as a chemoattractant in the down chamber being this result comparable to the 32% migration obtained by 0.5% LPS. These combined data indicate that AGP directly enhances EC motility as well as displays intrinsic chemotactic activity.

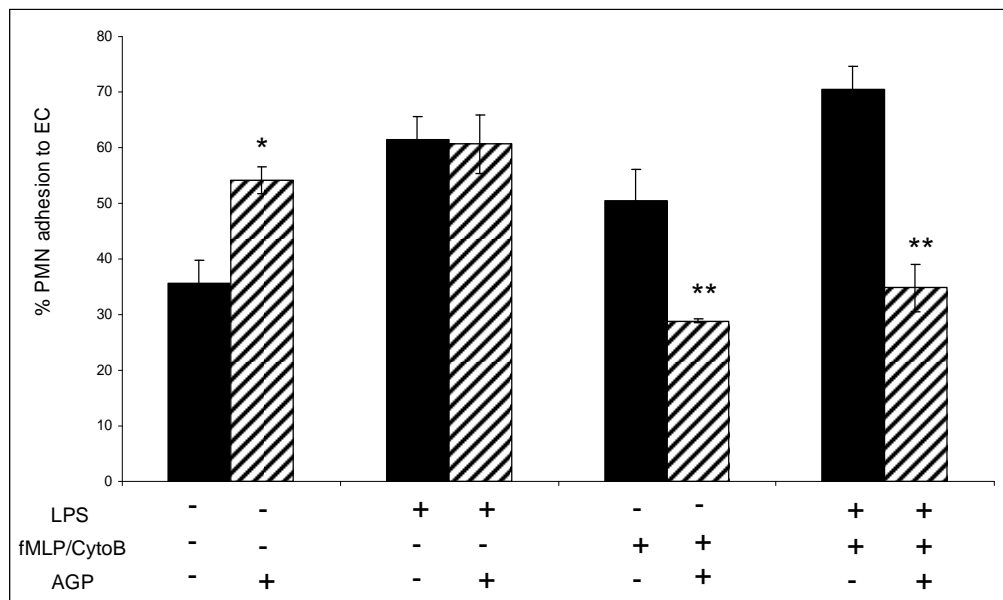


**Figure5. AGP Influences EC Motility:** EC were seeded at  $5 \times 10^4$  cells/chamber in the upper compartments of modified Boyden chambers in presence or absence of 0.5%FBS used as a positive chemoattractant control and/or the presence or absence of 0.75mg/ml AGP in the upper or bottom compartments. Results are expressed in fold migration using the non treated cells with no chemoattractant (media/media) as 1. Each bar represents mean ( $\pm$  SE) fold migration. \* indicates significantly increased compared to the simultaneous medium controls at  $p < 0.05$ .

**AGP Inhibits PMN Adhesion to the Endothelium:** AGP is highly sialylated and contains selectin-like carbohydrate moieties (Shiyan 1997). We asked whether AGP might modify adhesive interactions between PMNs and the endothelial



surface. Resting and stimulated PMNs were incubated with resting or activated endothelial monolayers and PMN-to-endothelium adhesion quantified (Figure 6). AGP increased adhesion of unstimulated PMNs to resting endothelia by 52%. In those experiments in which only endothelia was preactivated, AGP did not alter PMN-to-endothelium adhesion compared to the simultaneous control. In those experiments where PMNs were prestimulated in the presence or absence of endothelial preactivation, AGP diminished PMN-to-EC adhesion by 51% and 43%, respectively. The maximal inhibitory effect of AGP on PMN-to-endothelium adhesion (43% inhibition) was evident after both PMNs and endothelia were prestimulated. These data suggest that the ability of AGP to disrupt PMN-to-endothelium adhesion requires surface expression of adhesion molecules on PMNs that are only accessible after stimulation. Prestimulation of EC did not appear to be critical. Protein controls using BSA (Sigma) were performed to rule out any protein effect (data not shown).



**Figure 6. AGP Inhibits PMN Adhesion to the Endothelium:** Calcein AM-labeled PMN's stimulated with fMLP/CytoB or suspended in medium alone were incubated for 30min with resting or LPS-activated HMVEC-L monolayers in the presence (cross-hatched bars) or absence (closed bars) of AGP 0.75mg/ml. After washing, fluorescence of PMNs adherent to the HMVEC-L monolayers was measured. Each bar represents mean ( $\pm$  SE) % PMN adhesion (*i.e.* Adherent PMNs/Total PMNs \* 100%). \* indicates significantly increased compared to the simultaneous medium control at  $p < 0.05$ . \*\* indicates significantly decreased compared to the simultaneous medium control at  $p < 0.05$ .

## **Discussion and Conclusions**

AGP profoundly reduced capillary-like tube formation in a HMVEC-L system (fig.2). The presence of increasing AGP concentrations, altered HMVEC-L adhesion to matrigel in a biphasic manner (fig.3). AGP increased adhesion (+24.5%) of HMVEC-Ls at low concentrations (0.10mg/ml and 0.25mb/ml) whereas high concentrations of AGP (0.75mg/ml and 1mg/ml) were inhibitory (-29.7%). HVMEC-L migration in a wounding assay was reversibly inhibited (fig.4). Over 24h, AGP inhibited the migration into the wound by 44%. However, removal of AGP from the media permitted HMVEC-Ls to initiate migration into the wound reconstituting 60% of the wound by 12h, and 100% of the wound by 24h (fig.4). Serum induced HMVEC-L chemotaxis in a modified Boyden chamber was also influenced by 0.75mg/ml AGP, when AGP was used as a chemoattractant we saw a 25% migration, comparable to the 32% migration we obtained using FBS as a chemoattractant (fig.5). When AGP was used on the upper compartment, as a chemokinetic, migration increased by an 8%. When AGP was tested in the presence of FBS, we saw no difference whether AGP was tested in the upper or bottom chamber. When tested for PMN adhesion, AGP gave different results depending on PMN activation by fMLP/CytoB or not (fig.6). When neutrophils were not stimulated, adhesion was ~60% whether the endothelium was LPS activated or not, in presence of AGP. However, when the endothelium was activated, PMN adhesion diminished by ~50% in both activated endothelium and non. So again here, AGP seems to have a biphasic effect, increasing PMN adhesion of non stimulated PMNs and decreasing it when the neutrophils are activated independently of the LPS endothelial activation or not.

Angiogenesis is a key component of normal physiological process such as embryonic development, endometrial proliferation and wound healing (Brooks 1996). However, many pathological processes are also characterized by abnormal vascular development including arthritis, diabetic retinopathy and tumor growth and metastasis. We asked whether the APP, AGP, influenced new vessel formation. In our studies, AGP clearly inhibited Matrigel-induced angiogenesis of

HMVEC-Ls (fig.2). Irmak et al. performed both *in vitro* and *in vivo* angiogenesis assays using collagen I as a support and VEGF-A as an angiogenesis inducer (Irmak, Oliveira-Ferrer et al. 2009). They found that very low concentrations of AGP (300ng/ml) did not exert a pro-angiogenic effect. However, they found that AGP synergistically works with VEGF-A to increase angiogenesis. This study is not in conflict with our findings since in our experiments angiogenesis was promoted on Matrigel, in the absence of VEGF-A and the concentrations of AGP were substantially higher within the range concentration found in human serum during physiologic conditions.

Since *in vitro* angiogenesis is a multi-step process that includes adhesion to the underlying substrate, migration, proliferation and survival. Accordingly, we studied each step separately to establish whether one or more of them was influenced by AGP.

Matrigel is a gelatinous protein mixture secreted by Engelbreth-Holm-swarm mouse sarcoma cells that is mainly composed of collagen type IV, laminin and fibronectin (Arnaoutova, George et al. 2009). It resembles the complex extracellular environment found in many tissues. Endothelial cells bind to the extracellular matrix (ECM) through integrins (Giancotti 1999). Most integrins recognize multiple ECM proteins and conversely, individual matrix proteins bind to multiple integrins. Integrins  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  bind to collagens I, IV and laminin, whereas  $\alpha 3\beta 1$  binds mainly to laminin and fibronectin (Polverini 1996). AGP had a biphasic effect on adhesion, promoting it at low concentrations 0.10-0.25mg/ml and inhibiting it at higher ones 0.75mg/ml (fig.3). Additional studies on the effects of AGP on each HMVEC-L-expressed integrin are required to gain additional insight into the impact of AGP on the adhesion process.

On the wounding assay, AGP inhibited the migration (fig.4). When AGP was removed from the media cells started migrating into the wound, reaching a 100% confluence 24h after the AGP wash out was performed. Asserting both that the AGP effect was reversible, and that the AGP concentration used, 0.75mg/ml, did not damage the cells. We also performed viability tests that supported this latter statement (data not shown). On this wounding assay, HMVEC-Ls had to detach

from the neighbouring cells in the monolayer before they could start migrating into the wound.

To test the migration of single cells we performed a chemotaxis motility test. In this chemotaxis assay (fig.5), single cell suspensions were incubated in modified Boyden chambers where migration across a porous membrane was quantified. When FBS was used as a chemoattractant in the bottom chamber, no changes in migration were observed in presence of AGP, whether this was incubated in the upper or bottom compartment. AGP is known to bind and transport over 300 molecules (Israili and Dayton 2001), so we tested AGP alone, without the presence of FBS to see if FBS was interfering in AGP's activity. In this case we saw a slight chemokinetic activity when AGP was incubate in the upper chamber and a clear chemotaxis activity compearble to that exhort by LPS alone. This results support Irmak's paper were they report an increased endothelial cell migration even at very low AGP concentrations (5ng/ml-300ng/ml) (Irmak, Oliveira-Ferrer et al. 2009). This findings might be explained by the fact that during an acute phase response, new vessels might be needed to support he damaged tissue, and thus AGP acts as a chemotactic factor.

On the last set of experiments, AGP diminished adhesion of fMLP activated PMN's to the endothelium independently of it being LPS-activated or not. Activated PMNs bind to the endothelium mainly PSGL1, L-Selectin and glycosylated molecules that bind to E-Selectin, PSGL1 binds to P-Selectin through the terminal Sialyl LewisX (sLe<sup>x</sup>) and L-Selectin binds to the sialylated ligands on the endothelium. AGP has a high carbohydrate content that has a high affinity for E-selectin thus blocking this receptors for the PMNs to bind to (Lasky 1992). Plus the highly fucosylated and sialylated forms of AGP could compete with endothelial receptors in the adhesion process of the PMNs by blocking the L-selectin and the PSGL1 on the activated neutrophil.

## **FINAL REMARKS**

- Exogenous plasma boAGP binds to bovine neutrophils surface in an homogeneous way.
- High concentrations of boAGP down-regulate both spontaneous and ZAS induced exocytosis of secondary granules in bovine PMNs.
- When AGP is desialylated, the down-regulation activity is lost.
- Capillary-like tube formation of HMVEC-Ls on Matrigel is inhibited by hAGP.
- hAGP regulates HMVEC-L adhesion to Matrigel in a biphasic manner.
- Wound healing of HMVEC-Ls is inhibited by hAGP in a reversible manner.
- hAGP acts as a chemoattractant, in modified Boyden chambers for HMVEC-Ls.
- hAGP diminishes, PMN adhesion to endothelium when those are fMLP/CytoB activated.

## **FUTURE DIRECTIONS**

- In vivo studies for the angiogenesis inhibition.
- Binding studies with different integrins to resolve the biphasic behavior on the Matrigel adhesion
- Permeability assays, to see if the tightening of the monolayer is the cause of the wound healing inhibition since the chemotactic activity has already been proven.
- Further studies on human PMNs need to be done in order to fully understand the diminished adhesion.

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