Effects of adsorbed heat labile serum proteins and fibrinogen on adhesion and apoptosis of monocytes/macrophages on biomaterials

WILLIAM G. BRODBECK[1], ERICA COLTON[1], JAMES M. ANDERSON[1,2*]
[1]*Department of Pathology, Case Western Reserve University, Cleveland, OH, USA*
E-mail: wgb2@po.cwru.edu
[2]*Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, USA*

A previously established human monocyte culture protocol was used to determine the effects of varying adsorbed proteins on monocyte/macrophage adhesion and survival on dimethyl-silane (DM) or RGD modified glass coverslips. Cells were allowed to adhere for 2 h in the absence of protein or in the presence of serum, fibrinogen (Fg), heat inactivated serum (HIS), serum supplemented with Fg or HIS with Fg. Cell adhesion and apoptosis rates were determined on days 0 (2 h), 3, 7 and 10 of culture. The presence of serum alone in the initial culture was sufficient to optimize monocyte/macrophage adhesion and survival rates. Adding Fg to serum did not increase adhesion nor decrease apoptotic rates. No protein or the addition of HIS during the initial incubation period significantly decreased monocyte/macrophage adhesion and survival on both surfaces, however, the addition of Fg to HIS restored adhesion and survival rates to those seen with in the presence of serum alone on RGD surfaces. These studies demonstrate that monocyte/macrophage adhesion and survival on biomaterial surfaces are optimized by adsorbed heat labile serum proteins while adsorbed Fg plays a surface property-dependent role.

Introduction

Immediately following implantation, the surface of biomedical devices and prostheses is coated with a layer of plasma proteins. Surface adsorbed proteins are responsible for mediating monocyte adhesion to the implant by acting as ligands for cell surface integrin receptors. Once initial adhesion occurs, the adsorbed protein layer also contains proteins that can support the survival of monocytes and promote differentiation into macrophages and fusion into foreign body giant cells (FBGCs) [1–3]. Adherent monocyte/macrophages/FBGCs guide the foreign body response to implanted materials, which ultimately determines the biocompatibility of the device [4, 5]. Additionally, the types, levels and conformations of surface adsorbed proteins is dependent upon biomaterial surface characteristics [6], which in turn dictates adhesion and survival of monocytes and macrophages.

Previously, an established human monocyte protocol has been utilized to test the biocompatibility of a wide range of biomaterials by measuring monocyte adhesion and interleukin (IL)-4/interluekin (IL)-13-mediated macrophage fusion [7, 8]. However, the protocol utilizes autologous human serum, which contains significantly reduced concentrations of physiological relevant plasma proteins such as fibrinogen (Fg) and fibronectin (Fn). Using this system, we have previously shown that the adsorption of the inactivated form of the larger subunit of complement component 3 (iC3b) plays an integral role in initiating monocyte adhesion through interactions with monocyte cell surface integrin receptor, CD11b/CD18 in a surface-dependent manner [9]. However, these and other studies have implicated the importance of adsorbed Fg in mediating monocyte/macrophage adhesion also through cell surface CD11b/CD18 [10, 11].

To determine the relative importance of plasma and serum proteins in adsorbing to biomaterial surfaces and mediating monocyte/macrophage adhesion and survival, freshly isolated human monocytes were allowed to adhere to dimethyl silane and RGD modified glass surfaces in the presence of varying protein conditions. Since we have previously established a role for IL-4 in enhancing monocyte/macrophage survival [12] this cytokine was included in some cultures. We found that adsorbed serum proteins alone are sufficient to optimize monocyte/macrophage adhesion and survival on biomaterial surfaces and that heat labile serum proteins are essential components of serum necessary for adhesion. Furthermore, these studies demonstrate that Fg's role in promoting monocyte/macrophage adhesion and survival

*Author to whom all correspondence should be addressed. James M. Anderson, Institute of Pathology, Rm 306, Case Western Reserve University School of Medicine, Cleveland, OH 44106. USA.[1]

is more evident in the presence of Fn-derived RGD peptide.

Materials and methods

Preparation of surfaces

DM and RGD-modified glass coverslips were used as material surfaces. DM surfaces were chosen due to their previously described ability to promote monocyte/macrophage adhesion and survival [12], while RGD-modified surfaces were chosen to mimic the presence of adsorbed Fn.

DM surfaces were prepared as previously described [6]. RGD peptide (Sigma) was added to cleaned coverslips at 40 mg/ml in PBS and incubated and room temperature for 5 min followed by 2 PBS washes to produce the RGD modified surface. All surfaces were sterilized by heat sterilization and secured in 24-well plates by silicone rubber rings, which were sonicated in ethanol and autoclaved prior to use.

In vitro human monocyte culture

Whole blood was collected in the presence of 0.4% sodium citrate (Sigma, St. Louis, MO) from non-medicated, healthy adult volunteers. Human peripheral blood monocytes were isolated by a non-adherent density centrifugation method [9]. The cell culture medium consisted of RPMI-1640 supplemented with antibiotic/antimycotic and various proteins. The conditions consisted of either no protein, 20% autologous human serum, 20% autologous serum with a 20% plasma equivalent of purified Fg, 650 μg/ml (Enzyme Research Laboratories, South Bend, IN), 650 μg/ml Fg alone, 20% HIS, or 20% HIS with 650 μg/ml Fg. Isolated monocytes were plated at a concentration of 5×10^5/ml in 1 ml for triplicate surfaces. Following a 2 h incubation at 37 °C, surfaces were rinsed once with sterile PBS to remove any non-adherent cells and the media replaced. Media was also replaced on days 3 and 7, containing 10 ng/ml IL-4 (Sigma) where noted. Following the initial 2 hr incubation, and on days 3, 7 and 10, surfaces were removed and adherent cells stained with May-Grünwald /Geimsa or Annexin V-FITC.

Fibrinogen adsorption

To measure the amount of protein adsorption, a 10% concentration equivalent of ^{125}I-labeled Fg was used to spike the non-labeled Fg added to each culture well. After the 2 h incubation at 37 °C, surfaces were washed twice in PBS and radioactivity immediately measured. The radioactivity (in cpm) was then used to determine the mass of adsorbed Fg/surface area and is expressed as ng/cm^2.

Monocyte/macrophage adhesion

Adherent cell densities were determined following staining by May Grünwald/Giemsa. Surfaces were rinsed with PBS twice and adherent cells were fixed by the addition of methanol for 5 min. Cells were then washed with PBS and May Grünwald reagent was added for 1 min followed by another PBS wash. Giemsa reagent was added for 5 min followed by a final wash with dH_2O. Cell densities were determined from 5–20 × objective fields for each sample and are expressed as cells/mm^2.

Evaluation of apoptosis

Staining of adherent cells was performed with Annexin V-FITC reagent and percentage of apoptotic cells calculated as previously described [13].

Statistics

Data are expressed as the average of replicate experiments utilizing cells isolated from 3 different donors ± the standard error of the mean. Statistical comparisons were performed using ANOVA Bonferroni/Dunn test, using StatView v4.1 (Abacus Concepts, Berkeley, CA).

Results and discussion

As shown in Table I, fibrinogen adsorption was slightly, but not significantly ($p = 0.23$), decreased on RGD modified surfaces, indicating surface property-dependent adsorption, which corroborates previous studies [14, 15]. The levels of Fg adsorption were significantly decreased ($p \leq 0.023$) for both surfaces in the presence of serum and HIS. This phenomenon has been previously described and is most likely due to displacement of Fg by competition of proteins to adsorb to the limited number of available sites on the surface of the material (the Vroman effect) [16].

Monocyte/macrophage adhesion and apoptosis analyses on DM surfaces are shown in Fig. 1. The denaturing of heat labile serum proteins significantly reduced monocyte/macrophage adhesion to DM surfaces starting at day 0 and continuing throughout the remainder of the culture period (day 10) both in the presence and absence of IL-4 ($p \leq 0.040$ when compared to the serum control) (Fig. 1, Panel A). The addition of Fg to HIS during initial adhesion did not restore levels comparable to the control ($p \leq 0.030$). Furthermore, the addition of Fg to serum did not enhance adhesion beyond those levels seen in the presence of serum alone.

In conjunction with lower adhesion rates, cells initially cultured with HIS provided increased adherent cell apoptotic levels on DM surfaces at all time points

TABLE I Fibrinogen Adsorption

Surface	Condition	Surface density of adsorbed Fg (ng/cm^2)
DM	Fg Alone	156 ± 42
	Serum + Fg	58.8 ± 2.0
	HIS + Fg	68.9 ± 2.5
RGD	Fg Alone	116 ± 7.0
	Serum + Fg	46.2 ± 1.4
	HIS + Fg	58.8 ± 16

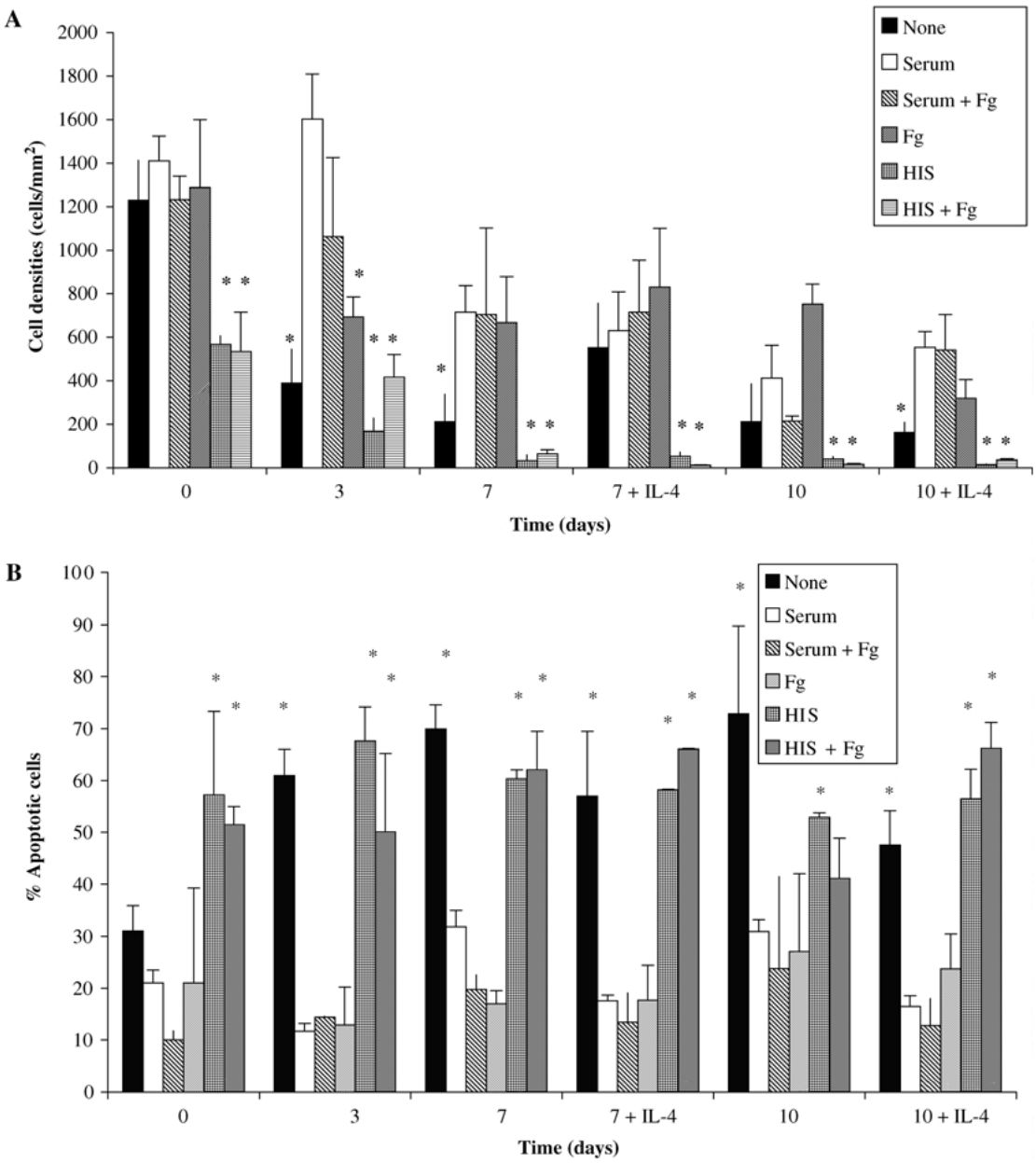

Figure 1 Protein adsorption effects on monocyte/macrophage adhesion and survival on DM surfaces. Isolated human monocytes were cultured with indicated proteins for 2 h. At the indicated time points, surfaces were washed and adherent cells stained with May Grünwald/Giemsa (Panel A) to determine cell surface densities or with Annexin V-FITC (Panel B) to determine apoptotic rates. Data represents the mean of three experiments using cells from different donors + sem. *Indicates significantly varying levels compared to serum control ($p < 0.05$).

(Fig. 1, Panel B), which was not reduced with the adsorption of Fg ($p \leq 0.041$ when compared to the serum control). Following day 0 of culture, cells allowed to adhere in the absence of any protein displayed significantly increased rates of apoptosis ($p \leq 0.021$). A similar mode of cell death has been previously described and termed intrinsic surface toxicity (IST) [17]. Therefore, the absence of heat labile serum proteins significantly reduced monocyte/macrophage adhesion and survival on DM surfaces while adsorbed Fg alone supported adhesion and survival but had no additive or synergistic effects when added to serum.

The effects of adsorbed proteins on monocyte/macrophage adhesion and apoptosis on RGD surfaces are shown in Fig. 2. Cell densities on the RGD surfaces remained comparable among the varying conditions until day 7 where cultures devoid of heat labile serum proteins during the initial incubation had significantly reduced adhesion rates ($p \leq 0.045$) (Fig. 2, Panel A). However, unlike on DM surfaces, Fg added to the HIS restored adhesion levels comparable to the serum control. Once again the presence of serum alone during initial adhesion established optimal adhesion, which was not enhanced by added Fg but was comparable to adsorbed Fg alone conditions.

As seen on DM surfaces, the absence of any protein or in the presence of heat labile serum proteins during adhesion lead to significantly increased levels of adherent cell apoptosis on RGD surfaces ($p \leq 0.035$) (Fig. 2, Panel B). However, in the presence of IL-4, Fg adsorbed with HIS restored monocyte/macrophage survival levels, supporting our previously described macrophage survival-promoting activities of IL-4 [12].

These studies demonstrate that adsorbed serum proteins alone are sufficient to optimize monocyte/macrophage adhesion and survival on biomaterial surfaces. Since no protein or the addition of HIS during the initial incubation period significantly decreased monocyte/macrophage

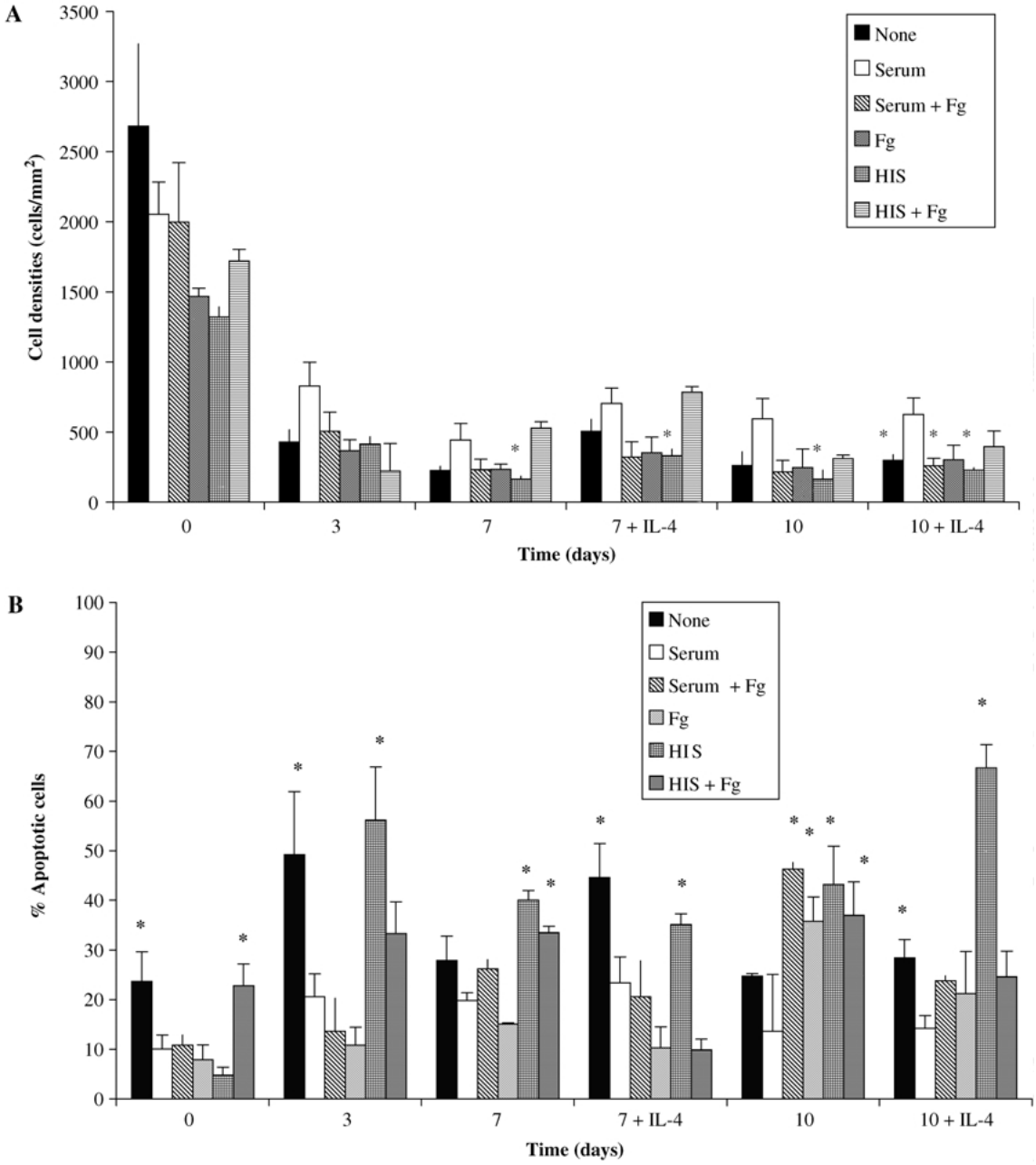

Figure 2 Protein adsorption effects on monocyte/macrophage adhesion and survival on RGD surfaces. Isolated human monocytes were cultured with indicated proteins for 2 h. At the indicated time points, surfaces were washed and adherent cells stained with May Grünwald/Giemsa (Panel A) to determine cell surface densities or with Annexin V-FITC (Panel B) to determine apoptotic rates. Data represents the mean of three experiments using cells from different donors + sem. *Indicates significantly varying levels compared to serum control ($p < 0.05$).

adhesion and survival on both surfaces, we conclude that heat labile serum proteins are essential components of serum necessary for adhesion. We have already demonstrated the integral role of iC3b in promoting monocyte/macrophage adhesion [9]. The addition of Fg to HIS restored adhesion and survival rates comparable to those seen with in the presence of serum alone on RGD surfaces, indicating that Fg's role in promoting monocyte/macrophage adhesion and survival is evident in the presence of adsorbed Fn-derived RGD peptide.

Acknowledgments

This work was funded through NIH grants HL-33849, HL-55714 and GM-20380 (fellowship awarded to WGB).

References

1. J. S. SUTTON and L. WEISS, *J. Cell Biol.* **28** (1966) 303.
2. A. R. MURCH, M. D. GROUNDS, C. A. MARSHALL and J. M. PAPADIMITRIOU, *J. Pathol.* **137** (1982) 177.
3. J. M. ANDERSON, in ''Biomaterials Science'', edited by B. D. Ratner, A. S. Hoffman, F. J. Schoen and J. E. Lemons (Academic Press, New York, 1996) pp. 451–455.
4. Q. ZHAO, N. TOPHAM, J. M. ANDERSON, G. LODOEN and C. R. PAYET, *J. Biomed. Mater. Res.* **25** (1991) 177.
5. Q. H. ZHAO, A. K. MCNALLY, K. R. RUBIN, M. REINER, Y. WU, V. ROSE-CAPRARA, J. M. ANDERSON, A. HILTNER, P. URBANSKI and K. STOKES, *ibid.* **27** (1993) 379.
6. C. JENNEY and J. ANDERSON, *ibid.* **49** (2000) 435.
7. A. K. MCNALLY and J. M. ANDERSON, *Am. J. Pathol.* **147** (1995) 1487.
8. K. M. DEFIFE, C. R. JENNEY, A. K. MCNALLY and J. M. ANDERSON, *J. Immunol.* **158** (1997) 3385.
9. A. K. MCNALLY and J. M. ANDERSON, *Proc. Natl. Acad. Sci. USA* **91** (1994) 10119.

10. L. TANG and J. W. EATON, *J. Exp. Med.* **178** (1996) 2147.
11. L. TANG, T. P. UGAROVA, E. F. PLOW and J. W. EATON, *J. Clin. Invest.* **97** (1996) 1329.
12. W. G. BRODBECK, M. S. SHIVE, E. COLTON and J. M. ANDERSON, *J. Lab. Clin. Med.* **139** (2002) 90.
13. W. G. BRODBECK, M. S. SHIVE, E. COLTON, Y. NAKAYAMA, T. MATSUDA and J. M. ANDERSON, *J. Biomed. Mater. Res.* **55** (2001) 661.
14. M. SHEN and T. A. HORBETT, *ibid.* **57** (2001) 336.
15. L. TANG, Y. WU and R. B. TIMMONS, *ibid.* **1998** (1998) 156.
16. S. M. SLACK, J. L. BOHNERT and T. A. HORBETT, *Ann. NY Acad. Sci.* **516** (1987) 223.
17. S. I. ERTEL, B. D. RATNER, A. KAUL, M. B. SCHWAY and T. A. HORBETT, *J. Biomed. Mater. Res.* **6** (1994) 667.

Received 1 October 2002
and accepted 15 January 2003