Constitutive neutrophil apoptosis in culture is modulated by cell density independently of β_2 integrin-mediated adhesion

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Abstract Although inflammatory mediators modulate the rate of constitutive neutrophil apoptosis in vitro the effects of micro-environmental conditions have not been fully investigated. In this study, we demonstrate that the rate of constitutive neutrophil apoptosis is affected by the number of cells per unit surface area, with enhanced survival at high cell density. Furthermore, the presence of protein or serum in the culture medium also enhances neutrophil survival. These effects were independent of β_2 integrin-mediated adhesion and were not influenced by specific adhesion to extracellular matrix components. Thus, the rate of neutrophil apoptosis is fundamentally influenced by micro-environmental conditions and indicates that factors such as cell density and extracellular protein concentration must be considered when investigating mechanisms regulating inflammatory cell apoptosis in vitro.

Key words: Neutrophil; Apoptosis; Adhesion; Micro-environment

1. Introduction

Tissue infection by pathogens initiates an acute inflammatory response characterised by rapid neutrophil recruitment [1,2] and release of neutrophil granule contents with anti-microbial activity to facilitate pathogen destruction [3]. Following elimination of the inflammatory insult, resolution of acute inflammation requires that recruited neutrophils are removed before they cause 'inappropriate' damage to tissue due to cytotoxic effects of granule contents [4–6]. Neutrophil apoptosis (programmed cell death) characterised by morphological and biochemical alterations, e.g. condensation and cleavage of nuclear chromatin [9] together with functional alterations that limit stimulus-driven release of cytotoxic granule contents [7,8] provides one mechanism for limiting the tissue destructive potential of the neutrophil. Furthermore, specific neutrophil membrane alterations signal macrophage recognition and phagocytosis of apoptotic neutrophils [10–12] via mechanisms which do not provoke pro-inflammatory mediator release, consistent with a role for apoptotic neutrophil death in resolution of inflammatory processes [13].

The rate at which neutrophils undergo apoptosis is likely to represent a critical factor determining the absolute tissue load of neutrophil products at inflammatory sites. The presence of inflammatory mediators such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and bacterial lipopolysaccharide (LPS) can prolong neutrophil longevity in vitro [14,15], and also potentiate neutrophil effector function. In addition, local environmental conditions such as hypoxia suppress the apoptotic programme in neutrophils [16] and it is likely that micro-environmental factors profoundly influence progression of the inflammatory response.

Recent studies indicate that in addition to the critical role of neutrophil adhesion in efficient recruitment of cells during development of the inflammatory response [17], adhesion plays an important role in regulation of cellular survival. Neutrophil apoptosis is potentiated following adhesion to fibronectin in a β_2 integrin-dependent manner when neutrophils are cultured on interleukin-1-stimulated endothelial cells [18]. A role for the β_2 integrin CD11b/CD18 (Mac-1) in the regulation of neutrophil apoptosis was further supported by studies in CD11b/CD18 deficient mice [19] where neutrophil apoptosis was found to be accelerated following engagement of CD11b/CD18 and assembly of the NADPH oxidase during phagocytosis. This pathway was suggested to provide a regulatory feedback mechanism for elimination of phagocytically active neutrophils, although these studies suggested that the constitutive rate of neutrophil apoptosis in suspension culture was largely CD18-independent. Furthermore, a recent study showing that cross-linking of CD11b/CD18 potentiated TNFα induced human neutrophil apoptosis again suggesting a role for β_2 integrins in the rate of activated neutrophil apoptosis [20].

In the present study, we have defined the environmental parameters which affect the rate of constitutive apoptosis of monolayers of neutrophils during in vitro culture. The rate of apoptosis was found to be decreased in a manner that is inversely related to the cell density per cm^2 of the culture vessel, apparently independent of adhesion to the culture vessel. In addition, neutrophil survival was enhanced when the cells were cultured in medium containing protein. The results of experiments presented in this paper together with our previous work raises the possibility that localised hypoxic conditions provide one mechanism for increased neutrophil survival at high cell density. Furthermore, our data demonstrate that careful consideration must be given to the design of culture conditions when studying the effects of agents that modulate apoptosis.

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Abbreviations: mAb, monoclonal antibody; MDM, modified Dulbecco's medium; db-cAMP, dibutyryl adenosine-3′,5′-cyclic monophosphate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine

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2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma, Dorset, UK except dexamethasone which was from David Bull Laboratories, Warwick, U.K. Cell culture media were from Life Technologies, Paisley, UK, dextran T-500 and Percoll from Pharmacia, Milton Keynes, UK, Diff Quick from Baxter Healthcare, Glasgow, UK and cell culture plastic was from Becton Dickinson, Leics., UK. Monoclonal antibodies (mAb): KIM 127, CD18 function promoting antibody [21] (gift from Martyn Robinson, Celltech); TS1-18, anti-CD18 (American Type Tissue Culture Collection); ICRF44, anti-CD11b [22] (gift from Nancy Hogg, ICRF); BBA-2, anti-CD62-E (R and D Systems, Oxon, UK); and VLA-3, anti-CD49c (clone 11G5; Serotec, Oxford, UK). Extracellular matrix components: fibronectin (Sigma); vitronectin (Calbiochem-Novobiochem, Nottingham, UK), and collagen types I and VI (Life Technologies).

2.2. Neutrophil isolation

Neutrophils were isolated from citrate anti-coagulated blood as previously reported [23]. Briefly, cells were separated from whole blood by centrifugation at $220\times g$ for 20 min and the upper platelet-rich plasma layer removed. Leukocytes were separated from the erythrocytes in the cell pellet by differential sedimentation using 0.6% (w/v) dextran T-500. Granulocytes were separated from the mononuclear cells through a discontinuous isotonic Percoll gradient. Granulocytes were harvested from the 63/73% interface of the gradient and were $>95\%$ neutrophils as determined by morphological analysis and $>99\%$ viable as determined by trypan blue dye exclusion.

2.3. In vitro culture of neutrophils

Neutrophils were suspended in Iscove's MDM containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and supplemented with either autologous serum (recalcified autologous plasma; 0.00008–2.4% (w/v albumin) corresponding to 0.001–30% (v/v serum)) or 0.001–0.1% (w/v) BSA. In a separate series of experiments, the neutrophils were suspended in Iscove's MDM containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and supplemented with either 10% (v/v) autologous serum or 0.1% (w/v) BSA, in the absence or presence of 2 mM db-cAMP or 1 μM dexamethasone. The neutrophils were dispensed into either flat-bottomed 96-well polypropylene plates or Teflon pots (Pierce, Chester, UK) at various densities and volumes and incubated at 37°C/5% CO_2.

2.4. Assessment of apoptosis

Triplicate cytocentrifuge preparations were made for each treatment and time point, stained with Diff-Quick, and apoptotic neutrophils identified by their darkly stained, condensed nucleus and cytoplasmic vacuolation [7]. In addition, neutrophils were labelled with FITC-conjugated CD16 mAb and samples analysed by flow cytometry with CD16 'low' expressing neutrophils representing the apoptotic population [23].

2.5. Antibody blocking experiments

Neutrophils were suspended at a density of 0.5×10^6/ml in Iscove's MDM supplemented with autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and dispensed into flat-bottomed 96-well polypropylene plates (0.1 ml/well) containing either PBS or test antibody (final concentration 20 μg of IgG/ml in PBS). After culture for 20 h at 37°C/5% CO_2, apoptosis was assessed morphologically.

2.6. Extracellular matrix

Extracellular matrix proteins (10 μg/ml) were adsorbed to flat-bottomed polypropylene 96-well plates (0.1 ml/well) overnight at 4°C and the wells washed with PBS. Neutrophils (1×10^6 cells/ml or 8×10^6 cells/ml) in Iscove's MDM supplemented with 10% (v/v) autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) were dispensed into the coated wells or uncoated, control wells (0.15 ml/well). The samples were then cultured for 20 h at 37°C/5% CO_2, after which the percentage of apoptosis assessed as described above.

2.7. Supernatant transfer experiments

Neutrophils were suspended at densities of either 0.5×10^6/ml (low density) or 4×10^6/ml (high density) in 4 ml Iscove's MDM supplemented with 10% (v/v) autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) then cultured overnight in Teflon pots. A positive control culture of high density neutrophils aged in the absence of serum and other exogenous protein supplements was also prepared. After culture for 20 h at 37°C/5% CO_2, the supernatants were collected and added to fresh neutrophils from the same donor at low density (0.5×10^6/ml). The serum-free supernatant was supplemented with serum to 10% (v/v), and a further control culture consisting of fresh neutrophils cultured in fresh medium supplemented with 10% (v/v) serum was prepared. The neutrophils were cultured for 20 h at 37°C/5% CO_2, after which triplicate cytocentrifuge preparations were made.

2.8. Statistics

All data are presented as mean ± S.E.M. for (n) separate experiments. Values were compared using the Student's t-test or where appropriate by one-way analysis of variance followed by the Newman-Keuls procedure. Differences were considered significant when $P<0.05$.

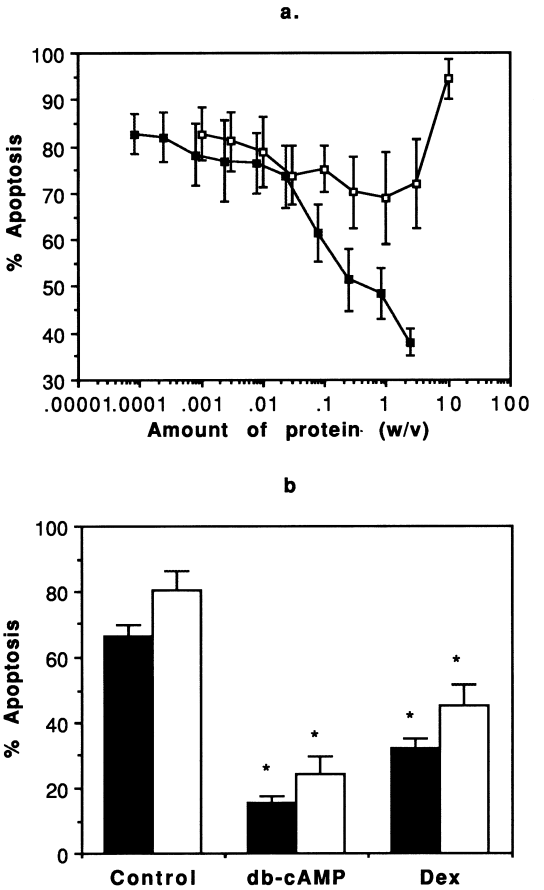

Fig. 1. The effect of serum and BSA on neutrophil apoptosis. Neutrophils (5×10^6/ml) were cultured for 20 h in flat-bottomed 96-well polypropylene plates after which the percentage apoptosis was assessed morphologically as detailed in Section 2. a: Neutrophils (0.2 ml) were suspended in the presence of either Iscove's MDM supplemented with 0.001–30% (v/v) serum (equivalent a serum albumin protein concentration of 0.00008–2.4% (w/v); closed symbols) or 0.003–10% (w/v) BSA (open symbols) (data represent mean ± S.E.M.; $n=5$). b: Neutrophils were suspended in medium supplemented with either 10% (v/v) serum (closed bars) or 0.1% (w/v) BSA (open bars) and cultured in the presence or absence of 2 mM db-cAMP or 1 μM dexamethasone (data represent mean ± S.E.M.; $n=5$; $P<0.05$).

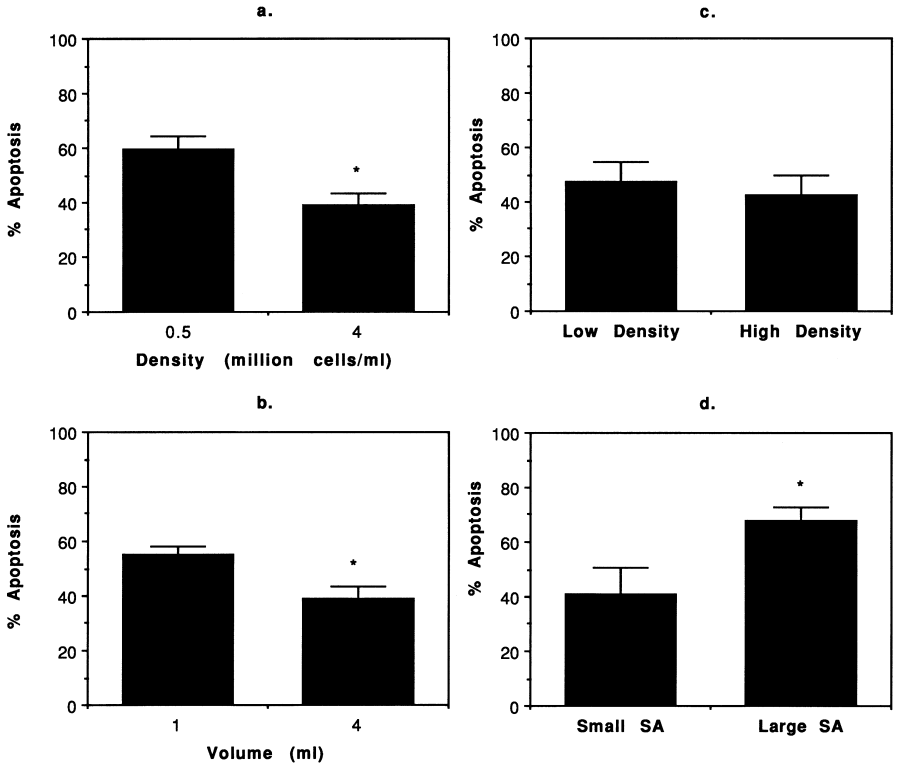

Fig. 2. The effect of volume, cell number and surface area on the rate of neutrophil apoptosis. Neutrophils were cultured for 20 h in Teflon vessels after which the percentage of apoptosis was assessed morphologically as detailed in Section 2. a: Neutrophils were suspended at either 0.5×10^6/ml or 4×10^6/ml in a constant volume of 4 ml (data represent mean ± S.E.M; $n=6$; $P<0.05$). b: Neutrophils were suspended at a constant density of 4×10^6/ml and cultured in volumes of either 1 ml or 4 ml (data represent mean ± S.E.M.; $n=4$; $P<0.05$). c: The total number of neutrophils cultured in each vessel was constant (16×10^6) but they were suspended at either a low density (32 ml) or a high density (4 ml) in large Teflon containers (data represent mean ± S.E.M.; $n=3$). d: Neutrophils (4×10^6) were cultured in Teflon containers with different surface areas. The surface area of the small and large containers were 201 mm^2 and 1385 mm^2 respectively (data represent mean ± S.E.M.; $n=5$; $P<0.05$).

3. Results and discussion

Unlike a number of other cell types, neutrophils undergo constitutive apoptosis when cultured in vitro [3]. The rate at which a population of neutrophils become apoptotic can be influenced by the addition of cytokines [14–16] or altered levels of second messengers within the cell [24] and by manipulating the extracellular environment [16]. However, in view of conflicting reports relating to the effects of certain mediators such as fMLP [15,25] and cAMP [26,27] on neutrophil apoptosis we have determined how the rate of neutrophil apoptosis is influenced by culture conditions.

We first assessed the effects of different serum concentrations upon constitutive neutrophil apoptosis during in vitro culture. In these experiments, neutrophils were cultured in the absence or presence of various concentrations of autologous serum. These experiments revealed that in the absence of exogenously added protein, neutrophils cultured for 20 h underwent secondary necrosis with large numbers of trypan blue positive cells present (data not shown). In contrast, the addition of 0.008–0.1% (w/v) concentrations of serum was sufficient to maintain neutrophil plasma membrane integrity, although a large proportion of cells underwent apoptosis (Fig. 1a). We next tested whether inclusion of bovine serum albumin (BSA) also provided survival in the absence of human serum components and whether survival was still augmented by db-cAMP or dexamethasone in the presence of BSA. As shown in Fig. 1a, addition of 0.1–1% (w/v) BSA was sufficient to rescue significant numbers of neutrophils from necrosis in the absence of other serum factors. However, in the presence of high concentrations of BSA (10% w/v) increased neutrophil apoptosis was observed. Neutrophil apoptosis in the presence of BSA could be inhibited by the addition of exogenous mediators suggesting that db-cAMP and dexamethasone survival effects are independent of other serum components (Fig. 1b). In contrast to the effects of high concentrations of BSA, serum (0.1–3% w/v) had a profound inhibitory effect upon neutrophil apoptosis suggesting the presence of additional survival factors. One possible explanation for the pre-survival effects of inclusion of protein in the culture medium is that respiratory burst activation and release of reactive oxygen species occurs in the absence of protein and engages 'activation-dependent' apoptosis pathways in neutrophils, accelerating the rate of apoptosis [28].

Having established that neutrophil survival factors in vitro required the presence of protein, but not necessarily serum, we next investigated the influence of the type of culture vessel. In this series of experiments, we cultured the neutrophils in the presence of 10% (v/v) autologous serum, allowing for direct comparison with other studies. Neutrophils were cultured for 18 h at various densities (0.5×10^6/ml or 4×10^6/ml) in Teflon containers. Results shown in Fig. 2a indicate that the rate of neutrophil apoptosis was inversely proportional to the cell density (density being defined as the number of cells per unit volume) suggesting that neutrophil survival was increased at high cell density. The observed density-dependent effect was

investigated further by varying the volume of cell suspension at a constant cell density in Iscove's MDM containing 10% autologous serum. Results presented in Fig. 2b suggested that the proportion of neutrophils undergoing apoptosis during in vitro culture was affected by volume. However, under these culture conditions neutrophils in suspension settle onto the surface of the culture vessel. When we attempted to keep cells in suspension they went necrotic suggesting a sensitivity to mechanical shear in vitro (data not shown). We then investigated the possibility that the absolute number of cells placed in a culture vessel of defined size may be of greater significance than either the cell density or the total volume of cell suspension. In order to test this suggestion we placed a constant number of cells in different volumes of culture medium in identical sized culture vessels. We found that under these conditions, the rate of apoptosis was unaffected (Fig. 2c) suggesting that cell concentration per se was not a critical factor. Since under these experimental conditions the surface area of the vessel was kept constant, we examined whether culturing a

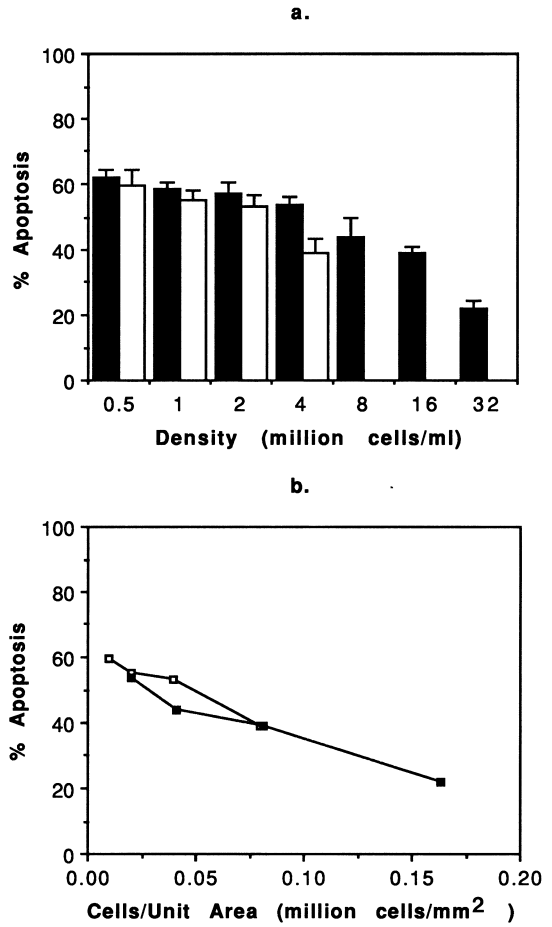

Fig. 3. Comparison of neutrophil apoptosis after culture in Teflon or polypropylene vessels. a: Neutrophils were cultured in Teflon (open bars) or polypropylene (closed bars) containers at a variety of different cell densities and a constant volume of 4 ml in Teflon containers and 0.1 ml in polypropylene containers. The percentage of apoptosis after 20 h was assessed morphologically as detailed in Section 2. Data represent mean ± S.E.M.; $n = 3$ separate experiments. b: The same data were expressed as the percentage of apoptosis per unit surface area of the culture vessel. The surface area of the Teflon and polypropylene containers were 201 mm^2 and 19.6 mm^2 respectively.

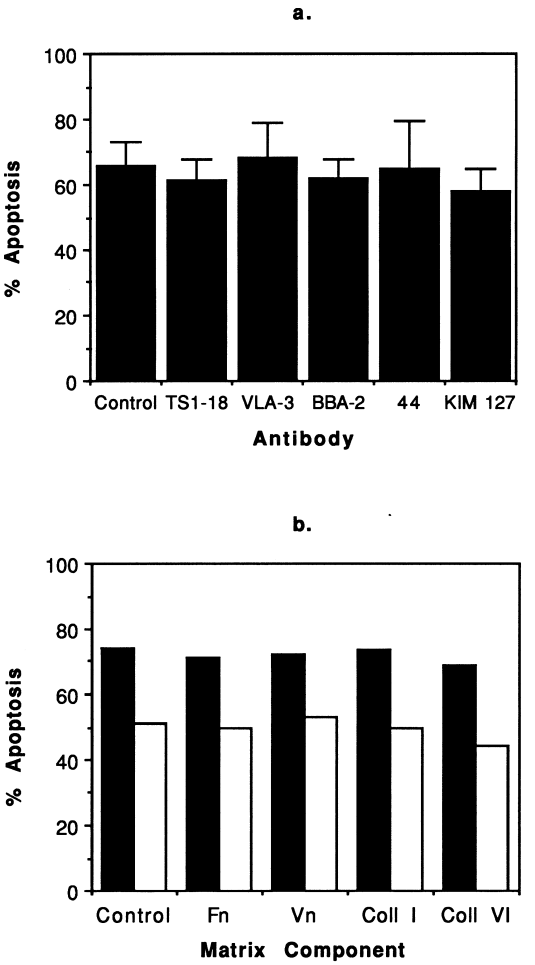

Fig. 4. The effect of culturing neutrophils in pre-coated polypropylene vessels. a: Neutrophils were cultured in polypropylene vessels for 20 h in the presence of a panel of antibodies as detailed in Section 2. The percentage of apoptosis after culture was assessed morphologically as previously detailed. Data represent mean ± S.E.M.; $n = 3$ separate experiments. b: Neutrophils were cultured in polypropylene vessels which were pre-coated with matrix proteins at low (closed bars) and high densities (open bars). The percentage apoptosis was assessed after 20 h of culture. Data show a representative experiment, which was confirmed by labelling with FITC-conjugated CD16 mAb as detailed in Section 2.

constant number of cells in vessels with different surface areas affected the rate of apoptosis. From results of experiments shown in Fig. 2d it was evident that the rate of apoptosis was inversely proportional to the number of cells per unit surface area. We next investigated whether cell density per unit area of culture vessel influenced the rate of apoptosis when cells were cultured on polypropylene, a surface to which neutrophils will adhere (data not shown). Although the density per unit volume of neutrophils which exhibit higher rates of apoptosis in polypropylene was higher than that in Teflon (Fig. 3a), when the data were expressed in terms of the number of cells per unit area (Fig. 3b) there appeared to be little difference between the two culture conditions, suggesting that adhesion to a culture vessel was not influencing the apoptosis rate under these culture conditions. Whilst the use of culture conditions that place neutrophils in contact with 2-dimensional surfaces to investigate regulation of apoptosis may fail to mimic the environment of neutrophils at inflammatory sites,

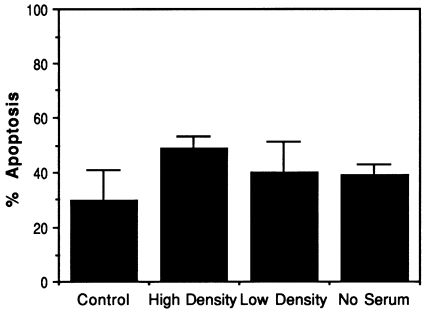

Fig. 5. The effect of culturing low density neutrophils in previously prepared supernatants from high and low density neutrophils. Neutrophils were cultured at 0.5×10^6/ml (low density) or 4×10^6/ml (high density) in the presence of 10% (v/v) serum or at high density in the absence of added serum in Teflon pots for 20 h as detailed in Section 2. The supernatants were collected and used as the culture media for fresh neutrophils from the same donor which were cultured at low density for 20 h. Control cultures containing fresh neutrophils cultured in fresh medium were also prepared. The percentage of apoptosis after culture was assessed morphologically as previously detailed (data represent mean ± S.E.M.; $n=3$).

these experiments establish that cell density per unit surface area represents a critical factor determining the rate of neutrophil apoptosis during in vitro culture.

In view of evidence that adhesion provides regulatory signals determining cell survival we next investigated whether specific adhesion via β_2 integrins affected the rate of apoptosis of neutrophils cultured in polypropylene. Neutrophils were cultured in the presence of CD18 blocking monoclonal antibodies (mAb). Although CD18 mAb efficiently blocked neutrophil adhesion to plastic in static adhesion assays (data not shown), we did not observe alterations in the rate of neutrophil apoptosis in the presence of CD18 mAb (Fig. 4a). Moreover, addition of the β_2 integrin function promoting mAb KIM 127 did not influence the rate of apoptosis, indicating that β_2 integrin-mediated adhesion events may not provide regulatory signals determining commitment to apoptosis in unstimulated cells. In addition, neutrophil apoptosis at either low (1×10^6/ml) or high (8×10^6/ml) cell densities was not altered by pre-coating the culture vessels with extracellular matrix proteins fibronectin, vitronectin or collagens (Fig. 4b). Together, these data suggest that engagement of specific adhesion receptors on neutrophils does not influence the constitutive rate at which neutrophils undergo apoptosis in vitro.

The possibility that neutrophils secrete factor(s) which at high cell density provide autocrine survival signals was addressed in a series of supernatant transfer experiments. Neutrophils were suspended at high and low densities in the presence of serum in Teflon pots and cultured for 20 h. The supernatants were collected and used to culture, at low density, freshly isolated neutrophils from the same donor (Fig. 5). Neutrophils cultured in the presence of supernatants from 'high density' neutrophils were not protected against apoptosis. Moreover, the supernatant from neutrophils cultured in the absence of serum did not cause any significant effect on the extent of apoptosis of fresh neutrophils cultured in vitro. Although cellular depletion of media constituents may complicate interpretation of these results, it seems likely that neutrophils are not capable of secreting survival factors at levels sufficient to rescue neutrophils cultured in vitro at low density from apoptosis.

In summary, examination of physical parameters which influence neutrophil apoptosis in culture revealed that survival is dependent on the number of cells per unit area of the culture vessel, consistent with the suggestions of Raff [29], that cellular communication is a key determinant of commitment to apoptosis. In addition, the presence of protein in the culture medium also augmented neutrophil survival. However, our data did not support a role for the principal neutrophil adhesion receptors, β_2 integrins, in providing regulatory signals that determine rates of apoptosis. Moreover, at low cell density, plating neutrophils onto a variety of extracellular matrix components failed to provide 'rescue' from accelerated apoptosis. Although supernatant transfer experiments do not exclude the possibility that high local concentrations of autocrine factors are present prior to harvesting of the supernatant, it is equally likely that high neutrophil density per unit area of culture vessel gives rise to local hypoxic conditions and pH changes which exert protective effects [16]. Furthermore, our findings that micro-environmental conditions influence neutrophil survival have important implications for study design when assessing the role of exogenous factors in regulation of the apoptotic programme in neutrophils.

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