Recombinant Human Albumin in Cell Culture: Evaluation of Growth-Promoting Potential for NRK and SCC-9 Cells *In Vitro*

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Abstract

Serum-derived albumin has for a long time been used in cell culture media, but the exact role of albumin and/or impurities bound to albumin has not been precisely defined. In this study, recombinant human albumin was evaluated for its growth-promoting activity on two cell lines, NRK and SCC-9. For NRK cells, the recombinant human albumin was found to exert an inhibitory effect. The fact that fatty acid free HSA was also inhibitory while HSA fraction V was stimulatory suggested a role for fatty acids or some other bound moieties in growth stimulation by HSA fraction V. Addition of oleic acid, cholesterol, phosphatidylcholine, phosphatidylserine or a combination of these lipids, however, did not significantly improve the growth stimulating activity of either fatty acid free HSA or the recombinant human albumin. For SCC-9 cells, both recombinant human albumin and fatty acid free HSA showed slight stimulation (although they were not as active as HSA fraction V), suggesting that in some cell systems, the albumin molecule *per se* may promote cell growth and survival.

Abbreviations: rHA = recombinant Human Albumin; HSA-faf = human serum albumin, fatty acid free; HSA-fr V = human serum albumin, fraction V; BSA-faf = bovine serum albumin, fatty acid free; BSA-fr V = bovine serum albumin, fraction V

Introduction

Mammalian cell culture has wide and expanding applications in research and in the biotechnology and pharmaceutical industries. These include the production of vaccines, cytokines and monoclonal antibodies for diagnostic and therapeutic purposes. Animal cell culture medium generally contains a small amount of serum, which is necessary to support growth and product elaboration (Ham and McKeehan, 1978). With the possibility of biological contaminants from serum (e.g. viruses and mycoplasma), the requirement to grow the cells in a defined medium, free from contaminants has become a high priority in the pharmaceutical industry. The use of even small amounts of human- or animal-

derived proteins in serum-free medium is coming under increasing scrutiny because of disease causing agents in both human- and bovine-derived materials (Hodgson, 1990). In addition, use of non-serum media allows for easier purification.

Albumin is a widely-used component of serum-free growth (Barlian and Bols, 1991; Ham et al., 1988; Hatzinger and Stevens, 1989; Drouin et al., 1987; Iscove et al., 1990 and Kiss, 1990) and production media (Jäger et al., 1988; Glassy et al., 1988). Due to the presence of hydrophobic pockets, its pliable structure and stability, albumin is especially suitable as a carrier of hydrophobic compounds. However, due to the strong binding sites on the albumin molecule, a completely pure preparation (free from other proteins

and low molecular weight contaminants) is difficult to obtain. Purity varies, generally between 96 to 99%, with many of the remaining contaminants being naturally bound ligands such as bilirubin, fatty acids, hormones, globulins and metal ions.

Addition of BSA to cell culture media has been shown to modulate the growth of different cells by different mechanisms. Long-chain fatty acids are quantitatively the most important component bound to albumin and have been found to be responsible for the growth promoting activity observed with albumin for many cell lines (Gershensen, 1967 and Nilausen, 1978). In these cases, other lipid binding molecules e.g. cyclodextrins and casein, have been loaded with lipids and used as replacements for BSA (Anderson et al., 1990; Szejtli, 1986).

Other hydrophobic ligands of albumin including bilirubin, erythropoietin-like factor, citrate and lysophosphatides, have been found to be responsible for the stimulation of growth associated with BSA for certain cell lines (Díaz-Gil et al., 1987; Congote, 1987; Kane, 1990, and Tigyi and Miledi, 1992).

It is, therefore, of interest to examine the activity of recombinant human albumin in cell culture systems which from earlier work (Keenan et al., 1996) had been shown to be responsive to bovine-derived albumin, in order to establish the possibility of replacing the bovine-derived albumin with recombinant human albumin. As the recombinant human albumin was produced in yeast using chemically defined raw materials, the absence of animal- and human-derived viruses and other related contaminants is assured and would allow for cell culture systems to utilise a safer, more defined and consistent raw material.

Materials and Methods

Tissue culture materials

DMEM and Ham's F12 media, trypsin, L-glutamine and sodium pyruvate were all obtained from Gibco Life Sciences; donor horse serum (DHS) was obtained from Flow Laboratories (29-211-54, lot 9120952); foetal calf serum (FCS) was obtained from Northumbria Biologicals (lot 41925); HEPES, BSA fraction V (A4919, lot 110H-04635), HSA fraction V (A1653, lot 86F-9383) and HSA fatty acid free (A3782, lot 119F-9303), oleic acid, cholesterol, phosphatidylcholine, phosphatidylserine, and paranitrophenol phosphate were all obtained from Sigma Chemical Company (Eng-

land); recombinant human albumin (lot GA91006) was obtained from Delta Biotechnology, Nottingham, UK; Ex-cyte III and BSA fatty acid free (82-042-2, lot 303) were obtained from Miles Diagnostics. The albumins, for which commercially delipidated forms were available, were of different batches to those used throughout the experiments; BSA fraction V (A4503, lot 12H0283), BSA fatty acid free derived from A4503 (A6003, lot 119F306), and HSA fraction V (A1653, lot 106F9333) and HSA fatty acid free derived from A1653 (A1887, lot 42H9313) were obtained from Sigma Chemical Company (England).

Cells

Normal rat kidney (NRK) cells were kindly provided by Ian Pragnell, Beatson Institute for Cancer Research, Glasgow, Scotland. SCC-9, a human squamous carcinoma cell line of the tongue was obtained from the American Type Culture Collection (CRL 1629). Cells were tested regularly for mycoplasma contamination and were found to be negative.

Growth assays

For NRK cells, growth stimulation assays were carried out using 24-well or 96-well plates in ATCC medium (1:1 volume DME:Ham's F12) and 1% donor horse serum (DHS) background. Albumin was tested in the range of 0.5–10 mg ml⁻¹, a range in which BSA fraction V had previously been found to be stimulatory for NRK cells. Cells were incubated for 3 to 4 days at 5% CO₂ and 37 °C.

Unless otherwise stated, all experiments were carried out three separate times and the average of the three sets of experiments were used in producing figures and tables.

For assays set up in 24-well plates, cell number or colony area using image analysis were the end points. For cell number, the cells in each well were trypsinised in a minimum volume of trypsin and counts were determined using a haemocytometer. Image analysis was used to measure colony area. After washing the wells three times in PBS A, a crystal violet stain (BDH 42555) at 0.25% v/v was used as a non-specific protein binding dye. This dye bound to the cell surface and allowed colony area to be measured using an image analyser (AMS 40-10 image analyser). The crystal violet dye was then eluted off the plates using 33% glacial acetic acid. The absorbance was measured at 570 nm and referred to as dye elution.

Acid phosphatase activity was used as the end point for assays set up in 96-well plates. From previous work (Martin and Clynes, 1991), acid phosphatase activity was found to exhibit a linear relationship to cell number.

Growth stimulation assays for SCC-9 cells were carried out in 96-well plates in ATCC medium and 0.25% FCS background. Albumin was tested in the range of 20–1000 $\mu\text{g ml}^{-1}$, a range in which BSA fraction V had previously been found to be stimulatory for SCC-9 cells (McGlinchy and Clynes, unpublished results). Cells were incubated for 7 days at 5% CO_2 and 37 °C. Acid phosphatase activity was used as the end point for assays set up in 96-well plates.

All albumins (excluding rHA) were reconstituted in sterile basal medium (DME:Hams F12 1:1 v/v) and sterile filtered. rHA was diluted from a 20% (w/v) solution in sterile basal medium. Ex-cyte III, a water-soluble cell culture nutrient, was diluted in sterile basal medium.

Albumin loading experiments

Albumin loading experiments were carried out using the method described by Jäger et al., 1988. Twenty microliters of an ethanolic solution of oleic acid (20 mg ml^{-1}) were slowly added per ml of rHA (50 mg ml^{-1} in PBS A) or HSA-faf (50 mg ml^{-1} in PBS A). The mixture was then allowed to complex overnight at 4 °C by gentle end-over-end rotation. The mixture was then filter sterilized and stored at 4 °C until use. Phospholipids and cholesterol were first dissolved in chloroform and then dried under a stream of nitrogen gas. Lipids were then dissolved in ethanol and complexed in the same manner and concentration as oleic acid.

Results

In order to evaluate the possible role of recombinant human albumin as a growth promoting factor and as a replacement for bovine serum albumin in cell culture media, rHA was compared to BSA-fr V, BSA-faf, HSA-fr V and HSA-faf.

Effect of recombinant human albumin on growth of NRK cells

Bioassays were set up using four end points to analyze results: acid phosphatase, image analysis, cell num-

Figure 1. Growth response of NRK cells to rHA using a variety of different end points: acid phosphatase, dye elution, cell number and image analysis. Results are expressed as the percentage growth relative to the control (2% DHS) $\pm$ SEM (n=3).

ber and dye elution (Fig. 1). The recombinant albumin consistently showed increasing inhibition at increasing albumin concentrations for all end points, although inhibition was much greater when image analysis was used as the experimental end point. With acid phosphatase, dye elution or cell number as the end point, a maximum inhibition of 50–70% was observed at 10 mg ml^{-1} , while inhibition of greater than 97% was seen at the same concentration, when using image analysis as the end point.

The difference in the extent of inhibition obtained using image analysis may reflect a combined inhibitory effect on cell growth and on cell spreading as these image analysis measurements were based on area. NRK cells are fibroblastic and do not form tight colonies in culture, but spread out over a relatively wide area. Indeed, after the stained colonies were measured using image analysis, the dye with which the cells were stained was eluted off the plate. The results obtained

Figure 2. Growth response of NRK cells to a variety of albumins (rHA, HSA-faf, HSA-fr V, BSA-fr V and BSA-faf). Results are expressed as the percentage growth relative to the control (2% DHS) $\pm$ SEM ($n=3$). Acid phosphatase content was used as the experimental end point.

were similar to those seen with acid phosphatase and cell number.

The effect of rHA on NRK cell growth was compared to the other albumins tested (Fig. 2). Like rHA, HSA-faf was inhibitory, showing increasing inhibition at increasing concentrations.

HSA-faf was less inhibitory than rHA, with maximum inhibition of about 20% at 2–10 mg ml⁻¹. HSA-fr V was growth stimulatory at increasing concentrations, reaching a maximum at 10 mg ml⁻¹. BSA-fr V was also stimulatory in a dose related fashion at a concentration range of 0.5 to 5 mg ml⁻¹. The BSA-faf was not as stimulatory, reaching a maximum of 50% stimulation over the control at 5 mg ml⁻¹ (Fig. 2).

When compared to HSA-fr V and BSA-fr V, it would appear that rHA was not a suitable replacement for BSA fraction V for NRK cells. However, the fact that the HSA-faf was also inhibitory would suggest that if fatty acids were loaded onto the albu-

min, then it might be possible to restore the activity. It should also be noted that while this batch of HSA-faf was inhibitory, BSA-faf was not. The difference between the human and bovine fatty acid free albumins may be due to batch-to-batch variation or species differences; the former explanation seems likely in view of the following results. Batches of Cohn fraction V HSA and BSA, different to those already used, were tested for their growth stimulating ability. The batches for which corresponding commercially available delipidated albumins were available, were tested for their growth stimulatory ability in comparison with the respective fatty acid free albumins. In this case, BSA-faf (A6003) was inhibitory, while HSA-faf (A1887) was mildly stimulatory; HSA-fr V (A1653) was stimulatory reaching 2.6-fold increase in growth stimulation at 5 mg/ml while the BSA-fr V (A4503) barely managed to increase growth by 0.4-fold in the same concentration range (Fig. 3). Significant variation may therefore occur in albumins prepared from plasma, possibly in the degree of removal lipid or stimulating factors.

Effect of recombinant human albumin on SCC-9 cells

SCC-9 cells were screened against the same batches of albumin initially tested on the NRK cells, in a concentration range of 20–1000 μ g ml⁻¹. A positive response was exhibited by the rHA in the range 20–500 μ g ml⁻¹ albumin (Fig. 4). However, the stimulation was not very high, with only 20–35% stimulation over the control. The standard deviation within each experiment was high (sometimes over 20%). The growth response did not appear to be linear. Above 500 μ g ml⁻¹, rHA appeared to lose some or all of its stimulatory ability. HSA-faf and HSA-fr V showed maximal stimulation at 1 mg ml⁻¹ and appeared to stimulate growth to the same extent (about 2.3-fold increase at 1 mg ml⁻¹). BSA-fr V and BSA-faf reached maximal stimulation at 100 μ g ml⁻¹ and 500 μ g ml⁻¹ respectively. BSA-fr V showed a slightly greater level of stimulation than that of rHA.

Addition of a lipid complex to enhance the activity of rHA on NRK cells

From the results obtained for NRK cells, it was observed that the fraction V albumins, both bovine and human sources, were more stimulatory than the respective fatty acid free albumins (Figs. 2 and 3). The possibility that the presence of fatty acids on the

Figure 3. Growth response of NRK cells to BSA HSA-fraction Vs and their respective fatty acid free albumins. Results are expressed as the percentage growth relative to the control (1% DHS) $\pm$ SEM (n=3). Acid phosphatase was used as the experimental end point.

albumin molecule could be a major contributor to the activity of the fraction V albumins was investigated by the addition of Ex-cyte, a commercially available lipid complex.

The addition of Ex-cyte did not increase the response of NRK cells to rHA or HSA-faf (Fig. 5). While the lower concentrations of Ex-cyte resulted in a 5–10% increase in growth for rHA and 3–5% increase for HSA-faf, this was not sufficient to offset the inhibitory activity of the rHA or improve the HSA-faf growth potential. The Ex-cyte was not stimulatory alone.

Lipid loading of recombinant human albumin

As the commercially available lipid-mixture was not stimulatory either alone or in conjunction with rHA or HSA-faf for NRK cells, loading of lipids onto the recombinant albumin was carried out in order to

Figure 4. Growth response of SCC-9 cells to a variety of albumins (rHA, HSA-faf (A1887), HSA-fr V (A1653), BSA-fr V (A4503) and BSA-faf (A6003)). Results are expressed as the percentage growth relative to the control (0.25% FCS) $\pm$ SEM (n=3). Acid phosphatase content was used as the experimental end point.

attempt to restore the stimulation seen with the fraction V albumins. Oleic acid, cholesterol, phosphatidylcholine and phosphatidylserine were chosen for analysis.

Without albumin as a carrier, none of the lipids exerted a significant stimulatory effect (Table 1). Cholesterol when complexed with rHA or HSA-faf improved the growth over the albumin alone but did not increase the activity sufficiently to offset the inhibitory effect of the albumin. Oleic acid when complexed with rHA or HSA-faf, resulted in some stimulation but this was not sufficient to neutralise the inhibitory effect of the albumin itself. At $10 \mu\text{g ml}^{-1}$, oleic acid complexed to albumin became very inhibitory. Phosphatidylcholine, when complexed to rHA or HSA-faf, showed no significant stimulation over the albumin alone. Various combinations of these lipids with or without albumin, showed little or no stimulation. The highest stimulation for the combinations occurred for

Table 1. Results are given as the average percentage growth relative to the control (1% DHS)±SEM (n=3) for all variables excluding phosphatidylserine and the combinations. For the remaining variables (phosphatidylserine and the combinations), the results are expressed as the average percentage growth relative to the control (1% DHS)±deviation of two separate experiments. Acid phosphatase was used as the experimental end point. In the table, Control Albumin refers to albumin that was exposed to end-over-end rotation overnight at 4 °C without being complexed to any lipid. The untreated albumin was not exposed to any treatment. The abbreviations for the lipid combinations are detailed in the legend below

		Growth as percentage of control		
			rHA	HSA-faf
Control				
Albumin			61.5±7.0	70.8±10.1
Untreated Alb			78.1±11.6	94.3±16.7
Lipids	$\mu\text{g ml}^{-1}$	No Albumin	rHA	HSA-faf
Cholesterol	1	76.7±8.8	67.8±4.1	83.5±13.7
	4	90.5±2.9	63.0±4.0	86.7±27.4
	10	90.8±8.9	64.3±5.4	90.4±27
Oleic acid	1	97.7±2.5	64.3±7.0	99.1±7.2
	4	97.6±10.6	63.1±6.6	103.0±20.1
	10	97.5±9.03	37.7±4.9	60.9±3.7
Phosphatidyl-choline	0.5	101.3±8.2	74.5±7.99	104.7±25.8
	1.0	101.5±9.7	78.2±11.8	102.0±24.0
	5.0	103.0±6.9	76.7±1.9	100.9±22.5
Phosphatidyl-serine	0.5	108.9±4.5	72.1±3.5	99.1±7.2
	1.0	109.7±12.9	66.4±6.8	103.0±20.1
	5.0	109.6±15.8	66.3±8.0	60.9±3.7
Combinations				
B		103.9±7.5	–	94.9±7.3
C		103.4±10.6	69.4±3.5	98.1±17
D		108.2±2.5	69.2±1.2	93.1±9.7
E		106.9±8.6	73.9±9.1	94.6±12
F		102.6±6.6	75.8±9.6	99.2±18
G		103.2±4.5	70.7±5.3	97.5±23
H		112.0±0.9	67.3±5.2	97.2±24
I		109.4±3.2	93.2±4.7	97.8±24
J		99.0±0.4	89.2±6.4	98.8±25
Legend: Abbreviations for lipid combinations				
Variables	Cholesterol $\mu\text{g ml}^{-1}$	Oleic acid $\mu\text{g ml}^{-1}$	Phosphatidyl- choline, $\mu\text{g ml}^{-1}$	Phosphatidyl- serine, $\mu\text{g ml}^{-1}$
B	4	4	–	–
C	4	4	1	–
D	4	4	1	1
E	–	4	1	1
F	–	4	–	1
G	4	–	1	–
H	4	–	–	1
I	–	4	1	–
J	–	–	1	1

Figure 5a. Growth response of NRK cells to varying concentrations of Ex-cyte III and varying concentrations of rHA. Results are expressed as the percentage growth relative to the control (1% DHS) $\pm$ SEM (n=3). Acid phosphatase content was used as the experimental end point.

combination I ($4 \mu\text{g ml}^{-1}$ oleic acid and $1 \mu\text{g ml}^{-1}$ phosphatidylcholine) with rHA. When compared to the untreated rHA (i.e. rHA that was not exposed to the conditions for lipid loading), there was a 19% increase in growth, which is not significant, given the variability inherent to the assay. For HSA-faf no such stimulation was seen.

Discussion

This paper reports one of the first studies on the effect of recombinant human albumin (rHA) in cell culture systems. The effect of rHA on the growth of two cell lines which had previously shown growth stimulation in the presence of BSA-fr V, was investigated. The

Figure 5b. Growth response of NRK cells to varying concentrations of Ex-cyte III and varying concentrations of HSA-faf. Results are expressed as the percentage growth relative to the control (1% DHS) $\pm$ SEM (n=3). Acid phosphatase content was used as the experimental end point.

recombinant albumin was found to be inhibitory to the growth of NRK cells, while being mildly stimulatory for SCC-9 cells. Of the albumins tested, the rHA should theoretically most resemble HSA-faf as it has the same amino acid structure and should contain none of the contaminants commonly found in association with fraction V albumins. HSA-faf was inhibitory to NRK cells and stimulatory to SCC-9 cells, so the rHA appeared to mimic the effect of HSA-faf. However, the extent of inhibition on NRK cells was more extensive and the stimulation of SCC-9 cells less than that for the HSA-faf.

The inability of rHA to stimulate the growth of NRK cells may have been due to a number of possibilities including: intrinsic inhibitory activity of the human albumin molecule for the NRK cells; the absence of some stimulatory serum-derived factor(s) normally present on fraction V albumins and on some delipidated albumins; inhibition due to some trace contaminant from processing or from the organism used to produce the recombinant albumin; the possibility that the highly purified recombinant albumin is sequestering essential components from the medium, for example

divalent ions (Mg^{2+} , Ca^{2+} and Cu^{2+}), amino acids (L-tryptophan and L-tyrosine), peptides or vitamins.

The albumin molecule itself may have some intrinsic inhibitory activity for NRK cells. In another series of experiments, BSA fraction V was fractionated using Heparin Sepharose™ chromatography. The bulk of the albumin obtained was found to be slightly inhibitory, or have no effect at all, while the stimulatory activity was contained in a fraction containing only 3% of the total albumin (Keenan et al., 1996). Further, fractionation of BSA using reverse-phase HPLC revealed the fraction containing albumin to be inhibitory (Keenan et al., 1995). Those results and the results obtained here, indicate that the stimulatory activity of the serum-derived albumins is associated with factors bound to the albumin rather than the albumin molecule itself.

The better growth of NRK cells observed with the fraction V albumins than with the 'purer' fatty acid free albumins suggested that the addition of lipids to rHA and HSA-faf may restore the activity seen with the fraction V. (Such a distinct observation could not be made for the SCC-9 cells.)

It was decided to combine the rHA and HSA-faf with lipids to see if stimulation as good as that seen with the fraction Vs could be obtained. Initially, a lipoprotein complex, Pentex Ex-cyte III was assayed in combination with rHA and HSA-faf. Ex-cyte is a water-soluble cell culture nutrient supplement derived from the lipid fraction of adult bovine or human sera which contains a mixture of lipoprotein, cholesterol, phospholipids and fatty acids. Although Ex-cyte has been found to support the growth and monoclonal antibody production of AHT-107 cells in a serum-free medium (Hewlett et al., 1989) and the growth of BHK-21 cells in reduced serum conditions (Smith et al., 1990), the addition of Ex-cyte did not restore the growth promoting activity to the levels observed with the HSA-fr V for either rHA or HSA-faf. Further, the Ex-cyte alone showed growth inhibitory activity in the system discussed here.

Directly loading some of the possible stimulatory factors (which might be present on serum-derived but not on recombinant albumin) onto rHA and HSA-faf did not enhance the activity. No significant stimulation by the lipids on their own was found nor to any significant extent when loaded onto the albumins. The concentrations used were unlikely to be too high as concentrations of $1-25 \mu g \text{ ml}^{-1}$ have been used in serum-free media (Jäger, 1988; Nilausen, 1978 and Miyasaki, 1991). It may be possible that some other

factors are responsible for the growth stimulation seen with the fraction V albumins.

Since albumin contains binding sites for a wide range of molecules in addition to fatty acids (e.g. L-tryptophan (Brown and Shockley, 1982), steroids (Kragh-Hansen, 1981), Cu^{2+} , Zn^{2+} , Ni^{2+} and Co^{2+} (Peters, 1970), citrate (Kane, 1990), lysolecithin (Nilausen, 1968), ecosanoids (Unger, 1972), glucose (Shaklai, 1984) and folate (Soliman and Olesen, 1976)), the possibility of any of these being responsible for the activity cannot be discounted. It is also possible that the highly purified albumin molecule may sequester essential components from the medium reducing the availability to the cells and hence retarding cell growth of NRK cells.

The presence of short-chain fatty acids which may be present on the rHA due to the cell type (yeast) in which it was produced and the manufacturing and purification methods used (i.e. use of octanoic acid as a stabilizer), may be relevant to its use in cell culture. Octanoic acid, at concentrations which might be present in albumin preparations, had only a marginally inhibitory effect on the cells, and could not, on its own, explain the inhibitory effect of rHA or HSA-faf (data not shown).

While bovine and human-derived albumins contain long-chain fatty acids (C_{16} to C_{22}), which are stimulatory to cells in culture, shorter chain fatty acids (C_{10} to C_{14}) are in general, inhibitory to mammalian cells (Ashbroke, 1972). Long-chain fatty acids have a greater affinity for albumin and would rapidly replace shorter chain fatty acids (Ashbroke, 1972, 1975). If such short-chain fatty acids were released into the medium, they may mask a growth stimulatory effect exerted by rHA. Conversely, the binding of long-chain fatty acids could contribute to the growth inhibitory effect exerted by rHA.

In conclusion, rHA was found to qualitatively mimic HSA-faf in being mildly stimulatory to SCC-9 cells but inhibitory to NRK cells. The inhibitory effect of rHA on the NRK cells did not appear to be predominantly a result of the origin of the albumin as an inhibitory response was also observed with HSA-faf. Lipid loading of rHA and HSA-faf did not restore the growth promoting ability to the levels observed for the fraction V albumins. The active factor would appear to be some contaminant of fraction V albumins which can be partially/fully removed or destroyed on delipidation. In all attempts to restore activity, rHA showed similar qualitative trends to HSA-faf. Future studies may reveal a variety of cell lines to which the rHA can

exert a stimulatory effect either alone or as a carrier of some lipid or other component.

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