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Short Communication

Opposite cytokine synthesis by fibroblasts in contact co-culture with osteosarcoma cells compared with transwell co-cultures

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ABSTRACT

We recently reported exchange of membrane and cytoplasm during contact co-culture between human Gingival Fibroblasts (h-GF) and SAOS-2 osteosarcoma cells, a process we termed 'cellular sipping' to reflect the manner in which cells become morphologically diverse through uptake of material from the opposing cell type, independent of genetic change. Cellular sipping is increased by Tumor Necrosis Factor-α (TNF-α), and we here show for the first time altered cytokine synthesis in contact co-culture supporting cellular sipping compared with co-culture where h-GF and SAOS-2 were separated in transwells. SAOS-2 had often undetectably low cytokine levels, while Interleukin-6 (IL-6), Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte–Macrophage Colony Stimulating Factor (GM-CSF) were secreted primarily by TNF-α stimulated h-GF and basic Fibroblast Growth Factor (FGF) was prominent in h-GF lysates ($p < 0.001$). Contact co-cultures permitting cellular sipping had lower IL-6, G-CSF and GM-CSF levels, as well as higher lysate FGF levels compared with TNF-α treated h-GF alone ($p < 0.05$). The opposite was the case for co-cultures in transwells, with increased IL-6, G-CSF and GM-CSF levels ($p < 0.03$) and no clear difference in FGF. We thus demonstrate significant phenotypic change in cultures where cellular sipping occurs, potentially contributing to tumor inflammatory responses.

1. Introduction

Malignant neoplasms comprise neoplastic malignant cells bearing multiple genetic mutations, together with supporting stromal cells amongst which fibroblasts are the most prevalent non-vascular cell type. Complex interactions between stromal and malignant cells are increasingly thought important in cancer [1–3], while we recently discovered a previously unknown interaction we have termed 'cellular sipping' [4].

In cellular sipping, there is exchange of membrane and cytoplasm between human Gingival Fibroblasts (h-GF) and malignant cells during contact co-culture. This generates morphological diversity amongst malignant cells and affected fibroblasts, without necessity for further genetic changes [4]. Morphology is the only phenotypic property reported as altered during cellular sipping [4], while we here investigate cytokine synthesis as a biologically relevant phenotype potentially influenced by cellular sipping. Although we reported cellular sipping across a range of melanoma and ovarian carcinoma cell lines, the process has been best characterized in SAOS-2 osteosarcoma cells where it is increased by pretreatment of h-GF with Tumor Necrosis Factor-α (TNF-α) [4].

TNF-α in malignant and stromal cells is associated with poor prognosis [5,6], while elevated levels of Interleukin-6 (IL-6) [7], Fibroblast Growth Factor (FGF) [8,9], Granulocyte Colony Stimulating Factor (G-CSF) [10], and Granulocyte–Macrophage Colony Stimulating Factor (GM-CSF) [11] are reported in some neoplasms. Notably, fibroblasts respond to TNF-α with increased cytokine synthesis [12–15], so that altered cytokine synthesis in culture conditions supporting cellular sipping may have particular bearing on stromal interactions with malignant cells in cancer. We here describe for the first time, different cytokine synthetic activity in h-GF in contact co-culture permitting cellular sipping, as compared with otherwise identical conditions preventing cellular sipping where cells are co-cultured separated in transwells.

2. Materials and methods

2.1. Isolation and culture of cells

h-GF were isolated by explant culture with informed written consent as approved by the Western Sydney Area Health Service Human Research Ethics Committee and grown in Complete

Abbreviations: BCS, bovine calf serum; BSA, bovine serum albumin; CM, complete medium; FGF, basic fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte–macrophage colony stimulating factor; h-GF, human gingival fibroblasts; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

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Medium (CM) comprising M199 (Sigma–Aldrich, St. Louis, USA), BCS (10%, Bovogen, Victoria, Australia) and antibiotics (all purchased from CSL Biosciences, VIC, Australia) penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) [4]. Experiments were with h-GF from 6th to 8th passage. The human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (ATCC, VA, USA) and propagated in CM as outlined previously [3,4]. Culture plasticware was from Iwaki, Scitech Division (Chiba, Japan), Sartorius, Ministart (Gottingen, Germany) and Costar (Cambridge, USA).

2.2. Contact co-cultures permitting cellular sipping

For experiments in tissue culture wells permitting cellular sipping, confluent h-GF were seeded in pre-gelatinised 12 well tissue culture plates. Once confluent, h-GF were further cultured with or without TNF-α (3 nM) purchased from Chemicon (Billerica, USA), for 24 h in M199 with antibiotics. SAOS-2 were seeded onto h-GF monolayers in M199 with antibiotics and BSA (4%, Sigma–Aldrich, St. Louis, USA) at 3.6×10^5 cells per well. h-GF and SAOS-2 cultured alone in M199 with antibiotics and BSA (4%) served as controls. Supernatants were collected at 24 h and centrifuged to remove debris, while lysates of monolayers were prepared by scraping attached cells into PBS with Triton-X 100 (0.5%, Sigma–Aldrich, St. Louis, USA). Supernatants and lysates were stored at −80 °C.

2.3. Transwell co-cultures preventing cellular sipping

Confluent h-GF were seeded above transwell 12 well inserts with a pore size of 0.4 μm for incubation in CM. Once h-GF monolayers were established, they were washed once with M199 and stimulated with TNF-α as in Section 2.2 above. Confluent SAOS-2 were seeded in M199 with antibiotics and BSA (4%) in lower transwell chambers so that h-GF were suspended above SAOS-2 on the porous transwell membrane. Controls comprised h-GF and SAOS-2 cultured alone in upper and lower transwell chambers respectively. Supernatants and lysates from upper and lower transwell chambers were collected separately at 24 h.

2.4. Enzyme Linked Immunosorbent Assays (ELISA)

ELISA were performed using Duoset® ELISA Development Systems from R&D Systems (Minneapolis, USA) according to the manufacturer's protocol, using serial dilutions of cytokine to establish standard curves. Streptavidin-HRP substrate reagents and sulfuric acid (H_2SO_4) were purchased from APS Chemicals (NSW, Australia). PBS tablets were acquired from Oxoid (Hampshire, England) while Tween-20 was from Sigma–Aldrich (St. Louis, USA) and ELISA plates were from Becton Dickinson Labware (NJ, USA). Absorbance was measured at 450 nm using a Bio-Rad Model 3550 micro-plate reader. Statistical analysis was by Student's *t* test.

3. Results and discussion

3.1. TNF-α increased h-GF expression of IL-6, GM-CSF G-CSF but reduced FGF

Unstimulated h-GF in isolated culture had very low and often undetectable levels of GM-CSF and G-CSF, whereas IL-6 was readily observed in supernatants as was FGF in lysates. TNF-α (3 nM) stimulation increased IL-6 ($p < 0.001$), GM-CSF ($p < 0.0001$), and G –CSF ($p < 0.0001$), and reduced FGF ($p < 0.0001$) (Table 1), consistent with the literature [12–15].

Table 1
Cytokine concentrations in supernatants and lysates of h-GF in isolated culture after 24 h of stimulation with TNF-α (3 nM) compared with unstimulated controls.

	Without TNF-α		With TNF-α	
Cytokine type	Supernatant (pg/ml)	Lysate (pg/ml)	Supernatant (pg/ml)	Lysate (pg/ml)
IL-6	672 ± 47.9	144 ± 8.9	971.17 ± 31.45*	516.29 ± 87.35
GM-CSF	18.17 ± 4.9	12.21 ± 7.81	221.15 ± 2.64*	86.90 ± 9.21
G-CSF	15.16 ± 9.14	16.60 ± 5.98	1178.45 ± 38.93*	148.85 ± 13.08
FGF	8.35 ± 6.17	1808.04 ± 37.69	276.81 ± 13.42	699.13 ± 38.88**

IL-6, GM-CSF and G-CSF were mostly in culture supernatants and increased upon TNF-α stimulation ($p < 0.001$)*, while FGF was primarily in lysates and reduced with TNF-α stimulation ($p < 0.0001$)**. Data are expressed as Mean ± SD of triplicate samples.

3.2. Co-culture experiments with h-GF pretreated with TNF-α (3 nM) maximized ability to evaluate cytokine synthesis in co-cultures

Fibroblast cytokine synthesis is well described [12–15], but our interest was to examine the effect of co-culture with SAOS-2 undergoing exchange of cytoplasm and membrane with h-GF during cellular sipping. Also, cellular sipping between SAOS-2 and h-GF is increased by TNF-α [4]. Thus all further experiments were performed with h-GF pre-treated for 24 h with TNF-α (3 nM) to maximize our ability to study tumor cell effects.

Fig. 1 shows the results of representative experiments comparing levels of cytokines studied in supernatants and lysates of TNF-α pre-treated h-GF alone, SAOS-2 alone and co-cultures of TNF-α stimulated h-GF with SAOS-2. Data is shown for contact co-cultures permitting cellular sipping, as well as for transwell co-cultures where cellular sipping was not possible [4]. Consistent with results in Table 1, levels of IL-6, G-CSF and GM-CSF were higher in supernatants than in cell lysates, while the reverse was the case for FGF.

3.3. Co-culture effects on cytokine levels reflected altered fibroblast rather than SAOS-2 synthesis

SAOS-2 had very low or undetectable levels of all cytokines studied in both supernatants and lysates. Also, supernatant cytokine levels were almost always appreciably higher in upper transwells containing h-GF compared with lower transwells with SAOS-2 (Fig. 1). We conclude that h-GF were the main source of cytokines measured in these culture systems, so that data reflect primarily h-GF responses to SAOS-2 co-culture.

3.4. Contact co-culture permitting cellular sipping reduced IL-6, G-CSF and GM-CSF levels in supernatants and lysates, but increased FGF in lysates

In contact co-cultures permitting cellular sipping between h-GF and SAOS-2 [4], supernatant levels of IL-6 ($p < 0.05$), GM-CSF ($p < 0.001$), G-CSF ($p < 0.0001$) and FGF ($p < 0.0001$) were consistently lower compared with TNF-α pre-treated h-GF alone (Fig. 1). A broadly similar pattern was seen in lysates, with lower levels of IL-6 ($p < 0.01$), GM-CSF ($p < 0.02$) and G-CSF ($p < 0.01$) upon contact co-culture (Fig. 1). A difference, however, was seen in FGF lysate levels, which were higher in contact co-cultures ($p < 0.001$). Similar results were obtained in three experiments using TNF-α pre-treated h-GF isolated from three donors.

3.5. Transwell co-culture preventing cellular sipping increased cytokine levels

When TNF-α pretreated h-GF and SAOS-2 were separated in transwell co-cultures to prevent cellular sipping [4], there were