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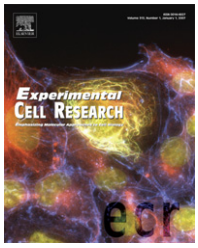

Research Article

Co-expression by keratinocytes of migration stimulating factor (MSF) and a functional inhibitor of its bioactivity (MSFI)

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ABSTRACT

Migration stimulating factor (MSF) is a potent autocrine and paracrine factor expressed by fibroblasts and epithelial cells in foetal skin, tumours and healing wounds. In tissue culture, MSF bioactivity is present in the conditioned medium of foetal and tumour derived fibroblasts, but not in normal adult fibroblasts or keratinocytes. The conditioned medium of early passage keratinocytes or a keratinocyte line (HaCaT) effectively inhibited the motogenic activity of rhMSF. Fractionation of keratinocyte conditioned medium by size-exclusion chromatography revealed the presence of bioactive MSF as well as a functional inhibitor of MSF (MSFI) in fractions corresponding to approximately 70 kDa and 25 kDa, respectively. MSFI was purified and identified as neutrophil gelatinase-associated lipocalin (NGAL or lipocalin-2). Immunostaining confirmed that keratinocytes expressed both MSF and NGAL, whereas normal adult fibroblasts did not express either. Recombinant and cell-produced NGAL neutralised the motogenic activity of rhMSF. NGAL is known to bind MMP-9 and promote the activity of this protease. In contrast, there was no evidence of NGAL-MSF binding in keratinocyte conditioned medium. MSF displays a number of bioactivities of relevance to cancer progression and wound healing. Our findings indicate a novel function of NGAL and a possible mechanism for regulating MSF activity in tissues.

Introduction

Migration stimulating factor (MSF; accession number AJ535086) is a truncated isoform of fibronectin, produced from the primary fibronectin gene transcript by a bypass of normal alternative splicing involving read-through of the intron separating exons III-1a and III-1b. This results in the inclusion of a unique 30 bp coding sequence, five in-frame stop codons and a cleavage/polyA signal. MSF protein is consequently identical to the 70 kDa N-terminus of fibronectin, up to and including the amino acid sequence coded by exon III-1a and terminates in a unique 10 amino acid sequence not present in any previously described "full-length" (250 kDa) fibronectin isoform [1]. Recombinant MSF (rhMSF) displays a number of potent bioactivities of relevance to wound healing and cancer development, including stimulation of cell migration, hyaluronan synthesis, angiogenesis (unpublished data [1–3]) and proteolytic activity [4].

MSF was first identified by its motogenic activity, present in the conditioned medium of cultured foetal fibroblasts. Foetal-

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skin fibroblasts migrate into 3-dimensional collagen gels to a significantly greater extent than their healthy adult counterparts [5]. This behavioural difference results from the production, by foetal cells, of a soluble migration stimulating factor, later identified as MSF [6]. Adult fibroblasts do not produce MSF but respond to it, so that when cultured in the presence of foetal fibroblast conditioned medium they display an elevated level of migration comparable to that of foetal cells. The retained responsiveness of adult fibroblasts provides the basis of a sensitive bioassay for assessing MSF production by cell lines in vitro and monitoring the purification of MSF from bioactive conditioned media [1,7,8].

Initial in vitro studies also demonstrated that tumour derived fibroblasts as well as paired forearm skin fibroblasts obtained from cancer patients behaved like foetal fibroblasts regarding their migration into collagen gels and production of MSF (reviewed in [2,3]). In contrast, early passage human keratinocytes and other epithelial cells did not migrate into collagen gels [9] and keratinocyte conditioned medium did not appear to contain MSF bioactivity (see results section).

MSF-specific antibodies have been raised against its unique C-terminal decapeptide and used to demonstrate that MSF is not normally expressed in adult tissues, but is transiently expressed during human acute wound healing and persistently over-expressed in a number of common human cancers (unpublished data, [1–3]) The localisation of MSF in tissue sections confirmed the results from in vitro studies regarding expression by fibroblasts. In contrast, the expression of MSF by epithelial cells highlighted a discrepancy between in vitro and ex vivo results: whilst there was no evidence of MSF production by normal epithelial cells in vitro, MSF was expressed by activated epithelial cells ex-vivo, including keratinocytes during wound healing [1,3].

Cells become activated when grown under standard tissue culture conditions, which include the presence of serum and other additives. Therefore, we hypothesise that keratinocytes in vitro should produce MSF and the reason for lack of MSF bioactivity in the conditioned medium may be the co-secretion of an inhibitor. The objective of this study was to test this hypothesis. Our results show that early passage human keratinocytes and HaCaT cells, a spontaneously immortalised keratinocyte cell line [10], constitutively express MSF in vitro. However, keratinocyte MSF is rendered inactive by the co-secretion of a functional inhibitor (MSFI). MSFI was isolated and identified as neutrophil gelatinase-associated lipocalin (NGAL or lipocalin-2). NGAL inhibits MSF-induced migration and controls the bioactivity of endogenous MSF by this mechanism. This represents a novel activity of NGAL, which does not seem to be mediated by previously reported mechanisms.

Experimental procedures

Reagents

Unless otherwise stated, reagents and chemicals were purchased from Sigma-Aldrich Company Ltd. (Gillingham, Dorset, UK).

Cell culture

A normal human fibroblast line (FSF44) was used in the migration assays. This line was established in our laboratory by explant culture from a foreskin biopsy, as previously described [5,6]. Experiments were performed with cells at passages 10–20. Early passage normal human keratinocytes (two lines tested) were a gift from Dr Fiona Watt [11,12] or purchased from ECACC (Sigma-Aldrich) and grown according to suppliers instructions. A spontaneously immortalised keratinocyte cell line (HaCaT) was a gift from Dr Norbert Fusenig [10]. Fibroblasts and HaCaT cells were routinely grown in MEM containing 15% (v/v) donor calf serum (Hyclone, Perbio Science, UK, Ltd., Cramlington, Northumberland, UK) and 2 mM L-glutamine (15% DCS-MEM) [5,6].

3D collagen gel migration assay

Type I collagen was extracted from rat tail tendons and used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as described [9]. Collagen gels in 35 mm tissue culture dishes were overlaid with 1 ml of serum-free MEM (SF-MEM) containing no further additives (baseline control) or the material to be tested (e.g. rhMSF, conditioned medium) at 4× the final concentration required. Confluent stock cultures of fibroblasts were trypsinised, pelleted by centrifugation, resuspended in growth medium containing 4% (v/v) donor calf serum at 2×10^5 cells/ml and 1 ml aliquots were added to the overlaid gels. Considering the 2 ml volume of gel, 1 ml medium overlay (with or without test materials) and 1 ml cell inoculum, this procedure gives a final concentration of 1% (v/v) serum (1% DCS-MEM) in both control and test cultures. Some experiments were performed using 5% serum (5% DCS-MEM) or serum-free conditions (SF-MEM). When tested, the same results were obtained in the presence or absence of serum. The assay cultures were incubated for 4 days and the percentage of fibroblasts found within the 3D gel matrix was ascertained by microscopic observation as described [13,14]. All migration stimulating and inhibiting activities were identified and quantified by this functional assay. Unless otherwise stated, putative inhibitors of MSF were added together with MSF and incubated for the duration of the assay.

In some experiments, the cells were pre-incubated with the MSF inhibitor prior to addition of MSF. The protocol in these cases was as follows: (a) Fibroblasts were plated onto the surface of the collagen gels at high density (2×10^5 cells in 1 ml of 4% DCS-MEM per gel). Over 90% of the cells attached and spread within 30 min. (b) Two hours after plating, 1 ml of SF-MEM with or without the inhibitor, at 4× the final concentration required, was added. (c) Following an incubation period of 1 h, 6 h or 24 h, the medium was removed and the cultures (controls and inhibitor-treated) were washed 6× with 1 ml of 1% DCS-MEM leaving 2–3 min between washes. (d) After washing, the medium was replaced by 2 ml of 1% DCS-MEM with or without MSF to give a final concentration of 100 pg/ml. (e) The number of cells migrated into the collagen gels was counted 4 days later. Further details are presented in result section.