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Original Paper

Human Malignant Fibrous Histiocytomas *In Vitro*: Growth Characteristics and Their Association with Expression of mRNA for Platelet-derived Growth Factor, Transforming Growth Factor-alpha and Their Receptors

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Eight human malignant fibrous histiocytomas were examined *in vitro*, in order to relate their growth properties to mRNA expression for platelet-derived growth factor (PDGF), PDGF receptor (PDGF-R), transforming growth factor-alpha (TGF-α) and the epidermal growth factor receptor (EGF-R). Reverse transcriptase–polymerase chain reaction (RT–PCR) showed that all cell lines expressed mRNA for PDGF-Rα and/or PDGF-Rβ; six cell lines expressed mRNA for the PDGF-A chain, with one cell line coexpressing PDGF-B chain mRNA; seven cell lines expressed mRNA for TGF-α whereas six cell lines expressed EGF-R mRNA. Conditioned medium from three cell lines contained PDGF; none of the cell lines released TGF-α. Two cell lines grew without serum requirements; whereas both expressed mRNA for PDGF, PDGF-R, TGF-α and EGF-R, other cell lines, unable to grow without serum, showed the same combination of growth factor/growth factor receptor expression. The two cell lines able to grow without serum were also shown to be stimulated by the addition of PDGF-BB. These findings show that simultaneous expression of mRNA for a growth factor and its receptor does not necessarily imply an autocrine or paracrine loop. However, two of our cell lines fulfil the requirements of possible PDGF-related autocrine and paracrine regulation. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: fibrous histiocytoma, cultured tumour cells, platelet-derived growth factor, transforming growth factor-alpha, messenger RNA, polymerase chain reaction, serum-free culture media, conditioned culture media

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INTRODUCTION

MALIGNANT FIBROUS histiocytoma (MFH) is a sarcoma, classified as a fibrohistiocytic neoplasia, and most often presenting as a soft tissue tumour [1]. There is a high risk of recurrence and a 5-year survival of 50–70% has been reported [2–5].

Few studies have dealt with the establishment and characteristics of human MFH *in vitro* [6, 7]. Whereas endogenous growth factors have been ascribed a role in the regulation of growth in other cell types [8–11], little is known about the significance of growth factors, such as platelet-derived growth factor (PDGF) or transforming growth factor-alpha (TGF-α) in MFH cells.

PDGF is a potent growth factor for connective tissue cells [12]. Consisting of two subunits, the A and B chains, PDGF can occur as three isoforms, PDGF-AA, PDGF-AB and PDGF-BB. The PDGF α-receptor (PDGF-Rα) binds all three isoforms, whereas the β-receptor (PDGF-Rβ) binds PDGF-BB with high affinity and PDGF-AB with lower affinity [13]. PDGF and its receptors are expressed in both human normal tissues and tumours, including gliomas and sarcomas, as well as in tumour cell lines [9, 14, 15]. The PDGF-B chain has been shown to be the normal, cellular homologue of the *v-sis* oncogene of a retrovirus, the simian sarcoma virus [16, 17].

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v-sis is able to transform the phenotype of cultured fibroblasts, and this altered phenotype can be reverted by adding antibodies to PDGF [18]. PDGF has, therefore, been ascribed a possible role in both the pathogenesis and the growth of tumours.

TGF-α is highly mitogenic for a variety of cells and has been demonstrated in both normal and neoplastic tissues [19, 20]. Belonging to the epidermal growth factor (EGF) family, it binds to the EGF receptor (EGF-R) [20, 21]. EGF-Rs are present in most human tissues [20] and elevated levels of EGF-R have been detected in several types of malignant cells, including MFH [21–23]. TGF-α has also been suggested to play a role in neoplastic transformation by autocrine or paracrine interactions with EGF-R [10, 24, 25].

This study was performed in order to investigate the growth characteristics of MFH *in vitro*, and to relate it to the expression of PDGF and TGF-α and their receptors, to investigate the possibility of autocrine and paracrine growth regulation.

MATERIALS AND METHODS

Patients

Tumour cell lines were established from 8 patients (age 37–84 years, mean 67 years) with MFH, undergoing surgery at the University Hospital, Linköping, Sweden (Table 1). Tissue specimens were obtained from either primary tumours or recurrences. 2 patients had received radiotherapy prior to surgery and subsequent cell line establishment.

Cell lines

The human osteosarcoma cell line U-2 OS [26] was kindly provided by Dr B. Westermark (Department of Pathology, University of Uppsala, Sweden). A cell line derived from a human giant cell tumour (Li-GCT 9; T. Walz, Department of Biomedicine and Surgery, Linköping University, Sweden) was available in our laboratory. Normal human foreskin fibroblasts (AG 1518) were obtained from the Human Cell Repository (Camden, New Jersey, U.S.A.).

Culture conditions

Fresh tumour specimens were immediately cut into blocks of approximately 1 mm^3 and cultured in 75 cm^2 cell culture flasks (Costar, Cambridge, Massachusetts, U.S.A.). Cells were grown in Eagle's Minimum Essential Medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics (penicillin (50 IU/ml), streptomycin (50 μg/ml)). The cultures were maintained at 37°C in humidified air containing 5% CO_2 and were subcultivated, using 0.25% trypsin, with a split ratio of 1:2 once or twice weekly. The established MFH cell lines will now be referred to as Li-MFH 1–8, respectively.

For culture under serum-free conditions, confluent cells (2×10^6 cells/flask) were washed four times with serum-free MCDB 105 culture medium [27] and kept for 48 h in MCDB 105 (10 ml/flask), supplemented with 0.1% bovine serum albumin, glutamine, and antibiotics as described above. The harvested conditioned medium was stored at −20°C. All tissue culture media and supplements were obtained from Life Technologies (Paisley, U.K.).

Cell doubling time

Cells were seeded in 35 mm dishes using Eagle's Minimum Essential Medium, supplemented as described above, including 10% FCS. Sets of two dishes were counted daily for 10 days, using a VDA 140 cell counter (Analys Instrument AB, Stockholm, Sweden).

Serum-free growth

To examine cell growth under serum-free conditions, a series of dishes received serum-free growth medium (MCDB 105, supplemented as above) 3 days after seeding, with subsequent daily cell counting as above.

DNA flow cytometry

The DNA contents of the cultured cells were determined as described previously [28, 29]. In brief, cells were detached from the culture flasks, suspended in citrate buffer and immediately frozen until processed. Suspensions of single-cell nuclei were stained with propidium iodide and analysed with a Leitz MPV flow cytometer (Leitz GmbH, Wetzlar, Germany). Histograms including at least 20 000 cells were recorded.

Primer synthesis

Each primer was designed to flank at least one convenient enzyme restriction site within the amplified product and/or to flank at least one intron/exon junction of the gene, in order to distinguish reverse transcriptase–polymerase chain reaction (RT–PCR) amplified RNA from genomic DNA and to identify transcript isoforms [30]. Forward (FWD) and reverse (REV) primers were synthesised commercially by Operon (Alameda, California, U.S.A.). The PDGF-A FWD primer corresponds to nucleotides 1329–1348 [31] and the REV primer to nucleotides 1066–1047 of the PDGF-A cDNA sequence [32]. The PDGF-A primer pairs could amplify both short (exons I–V + VIII) and long (exons I—VII) PDGF-A mRNA species [33]. The PDGF-B FWD primer corresponds to nucleotides 971–990 and the PDGF-B REV primer to nucleotides 1838–1819 of the PDGF-B cDNA sequence [34]. The PDGF-B primer pairs amplified across exons I–VII [33]. The PDGF-Rα FWD primer corresponds to nucleotides 3265–3285 and the PDGF-Rα REV primer to nucleotides 3530–3511 of the PDGF-Rα cDNA sequence [35]. The

Table 1. *The malignant fibrous histiocytoma (MFH) cell lines (Li-MFH* 1–8*) and corresponding patient data*

Cell line	Patient sex/age (years)	Tumour site	Primary tumour or recurrence	Radiotherapy (postoperative)
Li-MFH 1	F/70	Thigh	Primary	No
Li-MFH 2	M/73	Calf	Primary	No
Li-MFH 3	F/63	Retroperitoneal	Recurrence	Yes
Li-MFH 4	F/84	Upper arm	Recurrence	Yes
Li-MFH 5	F/73	Wrist	Primary	No
Li-MFH 6	F/37	Knee	Primary	No
Li-MFH 7	M/70	Thigh	Primary	No
Li-MFH 8	M/64	Back	Primary	No

PDGF-Rβ FWD primer corresponds to nucleotides 2805–2824 and the PDGF-Rβ REV primer to nucleotides 3317–3300 of the PDGF-Rβ cDNA sequence [36]. Primer design for PDGF-Rα and β was aided by the Primer 8 version 5 computer program (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, U.S.A.).

The TGF-α FWD primer corresponds to nucleotides 35–54 and the REV primer to nucleotides 216–197 of the TGF-α cDNA sequence [30]. The TGF-α primers amplified the intron–exon boundary between the sequences encoding amino acids 13 and 14 [30]. The EGF-R FWD primer corresponds to nucleotides 224–242 and the REV primer to nucleotides 444–426 of the EGF-R cDNA sequence [30]. The EGF-R primers amplified between amino acids − 6 and + 56 [30].

Intron differential RT–PCR (ID RT–PCR)

RNA isolation was performed by extracting total cellular RNA from cultures of Li-MFH 1–8, using the guanidium thiocyanate method [37]; cultures of the human cell lines U-2 OS, Li-GCT 9 and AG 1518 were used as positive controls.

Random-primed reverse transcription was performed using the SuperScript, RNase H^+-reverse transcriptase (Gibco, Gaithersburg, Maryland, U.S.A.), and according to the manufacturer's instructions. No more than 1 μg of total RNA was used for the reverse transcriptase reaction, which was carried out at 42°C for 60 min, followed by denaturation at 99°C for 5 min and then at 5°C for 5 min. The cDNA was subjected to 35 cycles of PCR, analysed by polyacrylamide gel electrophoresis and visualised by ethidium bromide staining, as described previously [30].

Determination of PDGF

PDGF in conditioned, serum-free medium was determined using an enzyme-linked immunosorbent assay (ELISA) kit specific for PDGF-AB (Amersham, Little Chalfont, U.K.). The assay, utilising a murine monoclonal antibody for PDGF-AA in conjunction with a polyclonal antibody for PDGF-BB, also recognises PDGF-AA and PDGF-BB (10% and 2% cross-reactivity, respectively), but not TGF-α, TGF-β, EGF, fibroblast growth factor (FGF) or tumour necrosis factor-alpha (TNF-α).

Determination of TGF-α

TGF-α in conditioned, serum-free medium was measured using an ELISA kit (Oncogene Science, Manhasset, New York, U.S.A.). The assay utilises affinity-purified goat polyclonal antibodies specific for human TGF-α; the antibodies had no cross-reactivity with EGF or with PDGF.

Determination of DNA synthesis stimulating activity in conditioned medium

DNA synthesis stimulating activity was determined essentially as described previously [38]. Briefly, AG 1518 human foreskin fibroblasts were seeded in 35 mm dishes using Eagle's Minimum Essential Medium, supplemented as described above, including 10% FCS. After 3 days, the medium was changed to serum-free MCDB 105 medium (2 ml/dish). After 48 h of serum starvation, the subconfluent cultures received conditioned medium or PDGF; the final volume of medium was kept at 2 ml/35 mm dish. At the same time, 0.2 μCi [^{3}H]thymidine was added per dish. After 48 h, the cultures were washed with water and treated with 10% trichloroacetic acid; the remaining material was solubilised in 0.3 M NaOH–1% sodium dodecyl sulphate. The incorporation of [^{3}H]thymidine was then measured in a liquid scintillation counter (1214 RackBeta, LKB Instruments, Surrey, U.K.).

Determination of growth stimulation by PDGF-BB

Li-MFH 1 and 5 cells were seeded in 35 mm dishes using Eagle's Minimum Essential Medium, supplemented as described above, including 10% FCS. After 3 days, the medium was changed to serum-free MCDB 105 medium (2 ml/dish). After 48 h of serum starvation, the cultures received PDGF-BB (final concentration 10 ng/ml medium). Cells were counted as above.

RESULTS

Growth characteristics

MFH cells grew readily *in vitro* and eight of eight consecutive cell lines were established (Table 2). The cell lines displayed a variety of morphologies at confluence, most showing elongated cells with an irregular shape, cartwheel formation and interspersed larger cells; cells were not

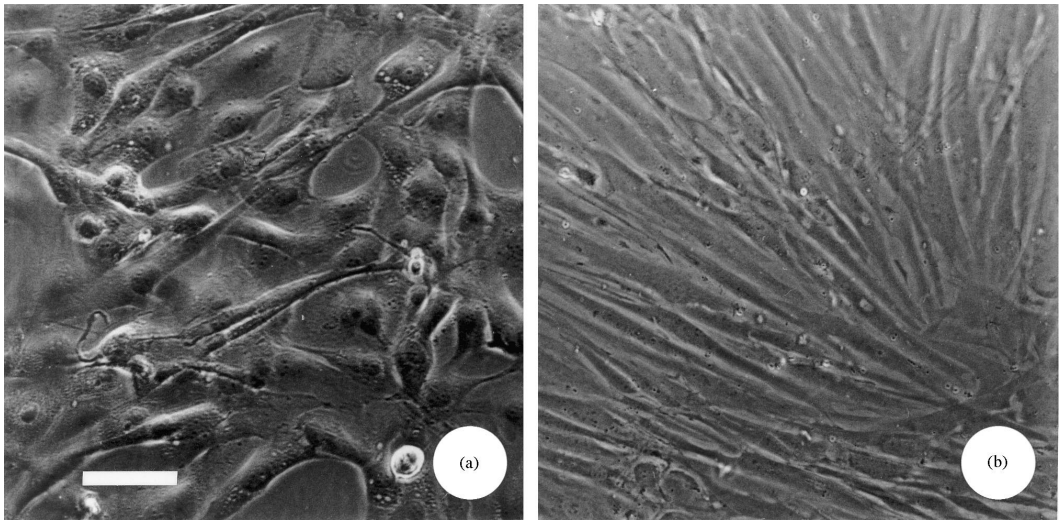

Figure 1. Photomicrograph showing the morphologies of Li-MFH 1 (a) and 4 (b) cells. The cell lines were grown in Eagle's Minimal Essential Medium, supplemented with 10% fetal calf serum, as described in Materials and Methods. Scale bar, 10 μm.

Table 2. In vitro *characteristics of Li-MFH* 1–8

Cell line	Ploidy	Serum requirement	Cell doubling time (days)
Li-MFH 1	2.70	–	2
Li-MFH 2	1.04	+	8
Li-MFH 3	2.52	+	ND
Li-MFH 4	1.00	+	ND
Li-MFH 5	1.53	–	2
Li-MFH 6	1.00	+	6
Li-MFH 7	1.09	+	3
Li-MFH 8	1.06	+	3

ND, not determined.

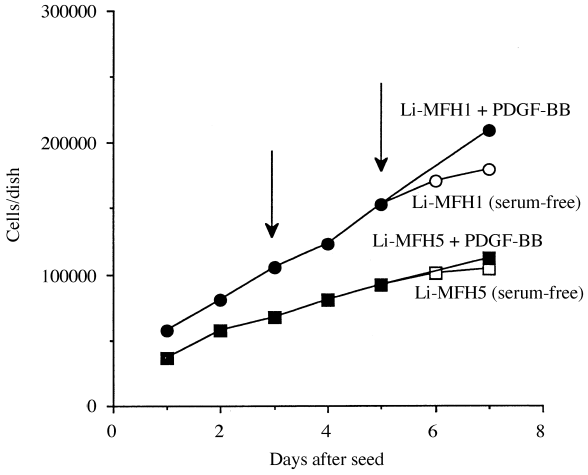

Figure 2. Cell growth of Li-MFH 1 and 5 under serum-free conditions and in the presence of PDGF-BB. Cells were seeded in the presence of 10% fetal calf serum. At day 3 the medium was changed to serum-free MCDB 105 (arrow). After another 48 h, sets of dishes received PDGF-BB (final concentration 10 ng/ml) (arrow). The data show that Li-MFH 1 and 5 cells are able to grow under serum-free conditions, and that they may become further stimulated by the addition of PDGF-BB. Each point represents the mean of two determinations.

confined to single layers. Li-MFH 4 cells were spindle-shaped and Li-MFH 1 and 5 cells roundish with a strong tendency to grow in clusters, as shown in Figure 1. The morphologies were unaltered throughout the subcultivations. Morphological heterogeneity has previously been reported in MFH cells of monoclonal origin and, thus, may be an inherent property of the cells [7]. Cell doubling times, as measured by cell counting, ranged from 2 to 8 days (Table 2).

Three cell lines, Li-MFH 1, 3 and 5, were aneuploid, with DNA contents ranging from 1.53 to 2.70, whereas the other cell lines, Li-MFH 2, 4, 6, 7 and 8 were diploid (Table 2). The levels and variability of DNA content were similar to those previously found in tissue specimens of soft tissue sarcomas [39].

Two cell lines, Li-MFH 1 and 5, did not require any addition of serum for growth, as shown by cell counting (Figure 2). The other cell lines failed to grow in the absence of serum.

Expression of mRNA

As demonstrated by RT–PCR, six cell lines, Li-MFH 1, 3, 4, 5, 7 and 8, expressed PDGF-A chain mRNA (Figure 3a), with one cell line, Li-MFH 1, also expressing mRNA for the PDGF-B chain (Figure 3b; Table 3). All cell lines expressed mRNA for both types of PDGF-R, except Li-MFH 8, which only expressed PDGF-Rα transcripts (Figure 3c,d). As expected, PDGF-A chain transcripts were found in U-2 OS human osteosarcoma cells, PDGF-B chain transcripts in Li-GCT 9 cells and transcripts for both types of PDGF-R in human normal AG 1518 fibroblasts. Six cell lines, Li-MFH 1, 2, 3, 4, 5 and 7 expressed both TGF-α and EGF-R mRNA (Figure 4; Table 3). Li-MFH 6 lacked both transcripts, whereas Li-MFH 8 expressed mRNA for TGF-α but not for EGF-R. The U-2 OS cell line, known to express transcripts of both TGF-α and EGF-R, was used as a positive control.

Release of growth factors into growth medium

Conditioned medium from Li-MFH 1, 4 and 5 was found to stimulate AG 1518 fibroblasts in a [^{3}H]thymidine incorporation assay (Figure 5). Medium from Li-MFH 1, 4 and 5 cells also

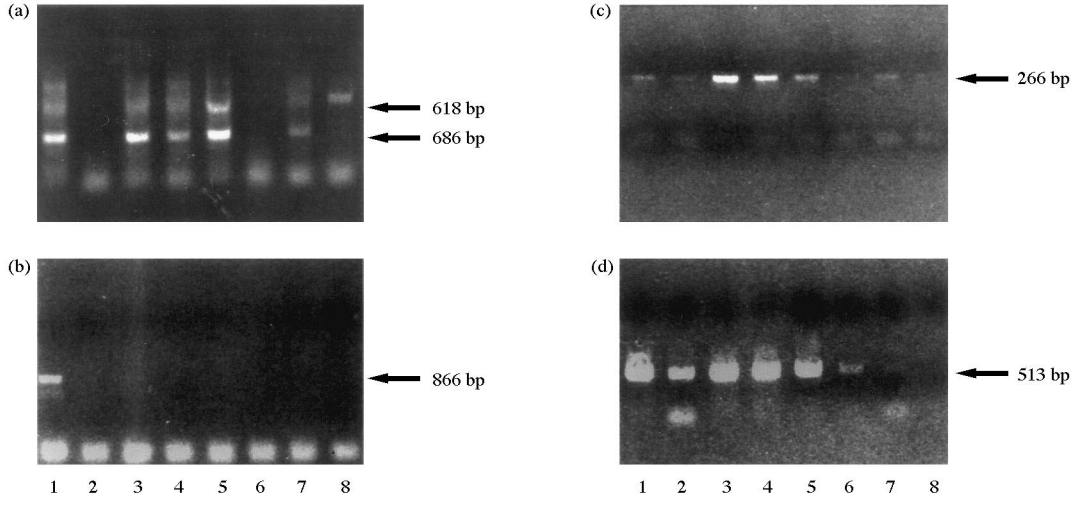

Figure 3. PDGF-A (a), PDGF-B (b), PDGF-Rα (c) and PDGF-Rβ (d) mRNA expression by cell lines Li-MFH 1–8, as demonstrated by reverse transcriptase–polymerase chain reaction (RT–PCR).

Table 3. *Expression of mRNA for platelet-derived growth factor (PDGF), transforming growth factor-alpha (TGF-α) and their corresponding receptors in Li-MFH* 1–8 *cell lines as determined by reverse transcriptase–polymerase chain reaction (RT–PCR)*

Cell line	PDGF-A	PDGF-B	PDGF-Rα	PDGF-Rβ	TGF-α	EGF-R
Li-MFH 1	+	+	+	+	+	+
Li-MFH 2	–	–	+	+	+	+
Li-MFH 3	+	–	+	+	+	+
Li-MFH 4	+	–	+	+	+	+
Li-MFH 5	+	–	+	+	+	+
Li-MFH 6	–	–	+	+	–	–
Li-MFH 7	+	–	+	+	+	+
Li-MFH 8	+	–	+	–	+	–

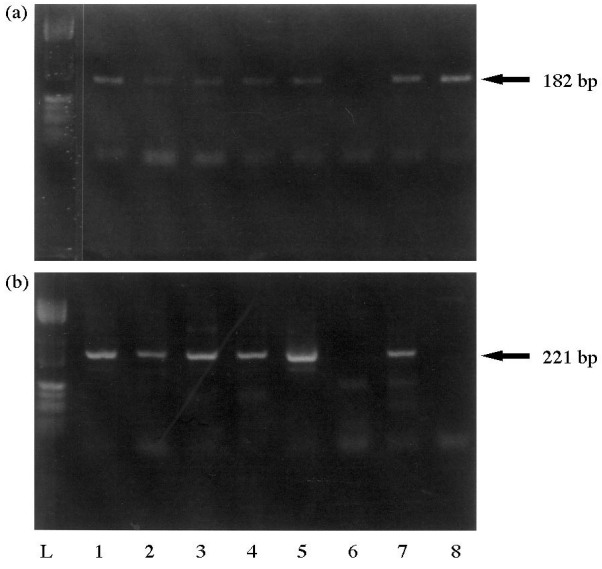

Figure 4. (a) Transforming growth factor-alpha (TGF-α) and (b) epidermal growth factor receptor (EGF-R) mRNA expression by cell lines Li-MFH 1–8, as demonstrated by reverse transcriptase–polymerase chain reaction (RT–PCR). L, molecular size marker.

contained PDGF-related material, corresponding to 3.8, 0.1 and 0.2 ng/ml of PDGF-AB, respectively (Table 4).

DISCUSSION

The observed heterogeneity of MFH *in vitro*, including the expression of mRNA for PDGF, PDGF-R, TGF-α and EGF-R, serum-free growth and secretion of PDGF into

Table 4. *Secretion of the growth factors PDGF-AB and transforming growth factor-alpha (TGF-α) into conditioned medium of the cell lines Li-MFH* 1–8, *as detected by using enzyme-linked immunosorbent assay (ELISA) kits, as described in Materials and Methods*

Cell line	PDGF-AB (ng/ml)	TGF-α
Li-MFH 1	3.8	0
Li-MFH 2	0	0
Li-MFH 3	0	0
Li-MFH 4	0.1	0
Li-MFH 5	0.2	0
Li-MFH 6	0	0
Li-MFH 7	0	0
Li-MFH 8	0	0

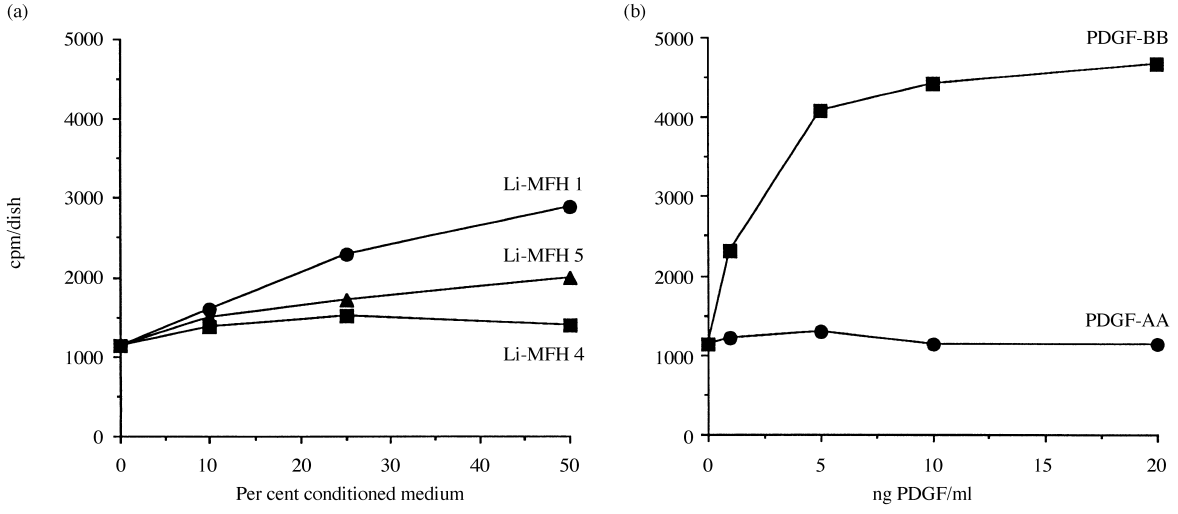

Figure 5. Effect of conditioned medium from cultures of malignant fibrous histiocytomas (a) and of platelet-derived growth factor (PDGF) (b) on [^{3}H]thymidine incorporation in AG 1518 human foreskin fibroblasts. Each point represents the mean of two determinations.

conditioned media, may reflect the previously described variability of *in vivo* morphology and behaviour of MFH.

The present results demonstrated that cultured MFH cells, at high frequency, expressed mRNA for PDGF, TGF-α and their receptors. Previous studies have shown expression of PDGF and TGF-α in several human malignancies, including soft tissue tumours, thereby ascribing them roles as putative autocrine growth factors [20, 40]. Coexpression of mRNA for PDGF and TGF-α and their receptors has not yet been described in cultured MFH cells.

Our findings that Li-MFH 1 and 5 cells, coexpressing PDGF and PDGF-R, were able to grow under serum-free conditions, suggest that they are regulated in an autocrine manner.

Our results also showed that Li-MFH 1, 4 and 5 cell lines released PDGF into the growth medium, ranging from 0.1 to 3.8 ng/ml PDGF-AB (Table 4), and stimulated the growth of AG 1518 fibroblasts. Assuming that the expression of the PDGF-B gene in Li-MFH 1 is reflected by the presence of PDGF-B containing peptides in the medium, and considering both known and presented dose–effect relationships of PDGF-AB [41] and PDGF-BB (Figure 5), a substantial part of the growth promoting activity (approximately 50% or more) could be ascribed to PDGF. In Li-MFH 4 and 5, lacking transcription of PDGF-B, PDGF-reactive material may be ascribed to PDGF-A molecules cross-reacting in the assay for PDGF-AB; according to the manufacturer, the cross-reactivity for PDGF-AA is 10%. However, PDGF-AA, being less potent than PDGF-BB in stimulating AG 1518 cells (Figure 5), may only account for a minor part of the growth stimulating activity in conditioned medium from Li-MFH 4 and 5. The remaining stimulating activity, therefore, should be ascribed to growth factors other than PDGF. The possibility that TGF-α contributed significantly to the growth promoting activity of the medium seems to be ruled out by the fact that no TGF-α was detected in the conditioned media from our cell lines (Table 4).

The significance of endogenous PDGF in Li-MFH 1 and 5 cultures is strengthened by the finding that the growth of these cells was stimulated by exogenous PDGF-BB (Figure 2).

However, in other MFH cell lines (i.e. Li-MFH 2–4 and 7), the combined expression of growth factor and its receptor was not coupled to serum-free growth and, therefore, the simultaneous expression of mRNA for a growth factor and its receptor is not generally indicative of an autocrine or paracrine mechanism.

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