Mitogenic Activity of Dermatofibrosarcoma Protuberans is Mediated via an Extracellular Signal Related Kinase Dependent Pathway

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Dermatofibrosarcoma protuberans is a malignant mesenchymal tumor originating in the dermis. Although it is locally aggressive and recurs unless completely excised, it only rarely metastasizes. In this study, we investigated the mechanisms of increased proliferation of dermatofibrosarcoma protuberans cells. The cells showed increased DNA synthesis in serum-free medium, which was demonstrated by the incorporation of [^{3}H]-thymidine. Increased DNA synthesis of dermatofibrosarcoma protuberans cells was abolished by genistein, a tyrosine kinase inhibitor, or by PD98059, a specific extracellular signal related kinase pathway inhibitor, but not by calphostin C, a protein kinase C inhibitor. Immunoblotting analysis of dermatofibrosarcoma protuberans cells using a specific antibody against phosphorylated extracellular signal related kinase (Thr^{202}/Tyr^{204}) showed that extracellular signal related kinase was expressed as constitutively phosphorylated molecules in dermatofibrosarcoma protuberans cells. Immunofluorescence analysis showed that the kinase was constitutively located in the nucleus of the cells. Furthermore, transfection of the dominant negative mutant extracellular signal related kinase into dermatofibrosarcoma protuberans cells abolished the increased mitogenic activity of the cells. These results suggest that an extracellular signal related kinase dependent pathway is implicated in the increased mitogenic activity of dermatofibrosarcoma protuberans cells. ***Key words: fibroblasts/kinase inhibitor/platelet-derived growth factor receptor/signal transduction. J Invest Dermatol 119:954–960, 2002***

Dermatofibrosarcoma protuberans (DFSP) is a rare malignant mesenchymal tumor that arises in the dermis and is characterized by latency in its initial detection, slow infiltrative growth, and local recurrence if not adequately treated (Laskin, 1992). Within the family of related fibroblastic tumors, the pathobiologic potential of DFSP is considered to be intermediate between that of dermatofibroma and malignant fibrous histiocytoma.

Deregulation of cell proliferation is an important event underlying the development of human neoplasia and can be determined by changes in factors controlling different steps in the cell growth process (Cohen and Ellewein, 1990). Growth factors acting through autocrine or paracrine mechanisms have been proposed to play an important role in cancer development and progression (Weinstein, 1987).

Recent studies reported that DFSP is characterized by specific cytogenic features: a translocation t(17; 22)(q22;q13) and supernumerary ring chromosomes derived from t(17; 22) (Pedeutour *et al*, 1993; 1996). Furthermore, Shimon *et al* reported that the DFSP-specific t(17; 22) translocation creates a molecular aberration by juxtaposing different portions of the α1(I)collagen (COL1A1) gene to the second exon of the platelet-derived growth factor b-chain (PDGFB) gene (Simon *et al*, 1997). The resulting chimeric gene encodes a COL1A1/PDGFB transcript of which the product contains the entire sequences for the processing of PDGFB. It is postulated that such rearrangements release the PDGFB from its physiologic control and unscheduled production of PDGFB creates an autocrine mechanism responsible for the development of DFSP. NIH 3T3 transformation by the rearranged human PDGFB gene is shown to produce a growth factor with autocrine and paracrine effects blocked by inhibitors of PDGFB activity (Greco *et al*, 1998).

PDGF isoforms are considered to be the principal mediators of mesenchymal cell proliferation, including dermal fibroblasts (Ross *et al*, 1986). Several studies suggest that the PDGFB signaling pathway is implicated in various neoplastic conditions (Robbins *et al*, 1982; Devare *et al*, 1983; Peres *et al*, 1987; Shamah *et al*, 1993; Carroll *et al*, 1996). Although PDGF-induced signal transduction pathways have been widely studied (Heldin *et al*, 1998), little is known about the signaling pathways utilized in DFSP cells. To further characterize DFSP cells, we investigated the mechanisms involved in their increased mitogenic activity. The results suggest that an extracellular signal related kinase (ERK) dependent pathway is implicated in the increased mitogenic activity of DFSP cells.

MATERIALS AND METHODS

Materials Genistein, PD98059, SB202190, and calphostin C, which were purchased from Calbiochem (La Jolla, CA), were dissolved in dimethyl sulfoxide (DMSO). Controls were incubated with an equal concentration of DMSO. The p44/42 ERK, phospho-specific ERK (Thr^{202}/Tyr^{204}) rabbit polyclonal antibodies, phospho-specific Elk-1

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Abbreviations: COL1A1, α1(I)collagen; DFSP, dermatofibrosarcoma protuberans; ERK, extracellular signal related kinase; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium.

(Ser^{383}), and purified Elk-1 fusion protein were obtained from New England Biolabs (Beverly, MA). Monoclonal anti-β-actin antibodies were purchased from Sigma.

Cell cultures The cultured DFSP tumor cells were derived from five patients in whom the diagnosis had been established both clinically and histologically, as described previously (Kikuchi *et al*, 1993). As controls, we used normal dermal fibroblast cell strains derived from normal skin obtained at the time of tumor resection. Dermal fibroblasts were also grown from explants of skin biopsies from five healthy donors, following institutional approval and informed consent. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Ihn *et al*, 1996; Ihn and Trojanowska, 1997; Ihn *et al*, 1997). Fibroblast cultures independently isolated from different individuals were maintained as monolayers at 37°C in an atmosphere of 5% CO_2 and 95% air (Ihn *et al*, 1996; 1997; Ihn and Trojanowska, 1997). Cells under the fifth subpassage were used for the experiments.

DNA synthesis The cells (2×10^4 per well) were plated in 24-plates in MEM with 10% FBS and grown to confluency, followed by 24 h incubation in MEM with 0.1% bovine serum albumin (BSA). Then, the cells were incubated with or without various concentrations of genistein, PD98059, or calphostin C for 24 h in the absence of serum and were labeled with [^{3}H]-thymidine (final concentration 1 μCi per ml; New England Nuclear, Boston, MA) for 2 h. The cell layers were washed three times with cold phosphate-buffered saline (PBS) and five times with ice-cold 5% trichloroacetic acid and dissolved in 500 μl of 0.1 N NaOH/0.1% sodium dodecyl sulfate. An aliquot of this extract was counted in a Beckman scintillation counter (Ihn and Tamaki, 2000a).

Transfections and constructs Transient transfections were performed as described previously (Ihn *et al*, 1996; 1997; Ihn and Trojanowska, 1997). Cells were transfected using a lipofection technique (FuGene™ 6 Transfection Reagent, Boehringer Mannheim, Indianapolis, IN) with various amounts of constructs (Ihn and Tamaki, 2000b; 2000c). The plasmid encoding the ERK2 [p42 mitogen-activated protein kinase (MAPK)] cDNA in which the Thr^{183} and Tyr^{185} required to be phosphorylated for activity were replaced with either glutaminic acid or alanine and phenylalanine, thus rendering the protein inactive (David *et al*, 1995; Ihn and Tamaki, 2000a). pSV-β-galactosidase control vector (Promega, Madison, MI) was transfected to visualize transfection efficiency. The cells were maintained in serum-free MEM and 0.1% BSA for 24 h and the DNA synthesis was determined as described above.

Immunoblotting For the preparation of cell lysates from the cultured DFSP tumor cells and normal dermal fibroblasts, cells were placed in MEM and 0.1% BSA for 24 h. Then, the medium was removed and the cells were washed with PBS. The cells were lyzed by scraping into a solubilization buffer [50 mM Tris–HCl, pH 8, 150 mM NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA), 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg per ml aprotinin, 10 μg per ml leupeptin, and 10 μg per ml pepstatin]. The lysates were incubated at 4°C for 30 min and then centrifuged for 5 min at 4°C. Protein concentrations of lysates were determined using a Bio-Rad (Hercules, CA) protein assay, as recommended by the manufacturer. Immunoblotting was performed as described previously (Ihn and Trojanowska, 1997; Ihn and Tamaki, 2000a; Ihn *et al*, 2001). Briefly, cell lysates (20 μg) obtained from cells were subjected to electrophoresis on 10/20% gradient sodium dodecyl sulfate polyacrylamide slab gels, and then electrotransferred from the gels onto nitrocellulose sheets. The nitrocellulose sheets were incubated overnight with the indicated primary antibodies. Bound antibodies were detected with horseradish-peroxidase-conjugated antirabbit IgG and immunoreactive bands were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL) as described previously (Ihn and Trojanowska, 1997; Ihn and Tamaki, 2000a).

Immunofluorescence Immunofluorescence analysis was performed as described previously (Shuai *et al*, 1992). In brief, the cultured DFSP tumor cells and normal dermal fibroblasts were cultured in eight-well tissue culture chamber slides. After cells were placed in MEM and 0.1% BSA for 24 h, the medium was removed and the cells were washed with PBS and fixed in 3.7% formaldehyde/PBS for 10 min. After two washes with PBS, cells were incubated for 30 min in PBS containing 10% FBS. Cells were permeabilized with 0.5% Triton-X. An antibody against ERK was added and the slides were incubated for 1 h at 37°C. After three washes, a fluorescein-conjugated secondary antibody was added and the slides were incubated for 1 h at 37°C.

ERK activity assays Kinase assays were performed as described previously (Chin *et al*, 1999) with minor modifications. Briefly, cells were lyzed in buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg per ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined as described for immunoblotting. Total protein (200 μg) samples were incubated with phospho-specific ERK (Thr^{202}/Tyr^{204}) rabbit polyclonal antibody overnight on a rocker at 4°C. Protein A-Sepharose beads were then added to immunoprecipitate the activated ERK complex. The immunoprecipitate pellets were incubated with 1 μg of Elk-1 fusion protein in the presence of 100 μM ATP and a kinase buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. The reaction was terminated with sodium dodecyl sulfate loading buffer. The samples were analyzed on 10/20% gradient sodium dodecyl sulfate polyacrylamide slab gels as described in *Immunoblotting*. ERK activity was assayed by detection of phosphorylated Elk-1 using a phospho-specific Elk-1 rabbit polyclonal antibody (1:1000). After overnight incubation with the primary antibody at 4°C, the membrane was incubated for 1 h with a horseradish-peroxidase-conjugated antirabbit secondary antibody. The proteins were subsequently detected using LuminoGLO (New England Biolabs) as described previously (Ihn and Trojanowska, 1997; Ihn and Tamaki, 2000a).

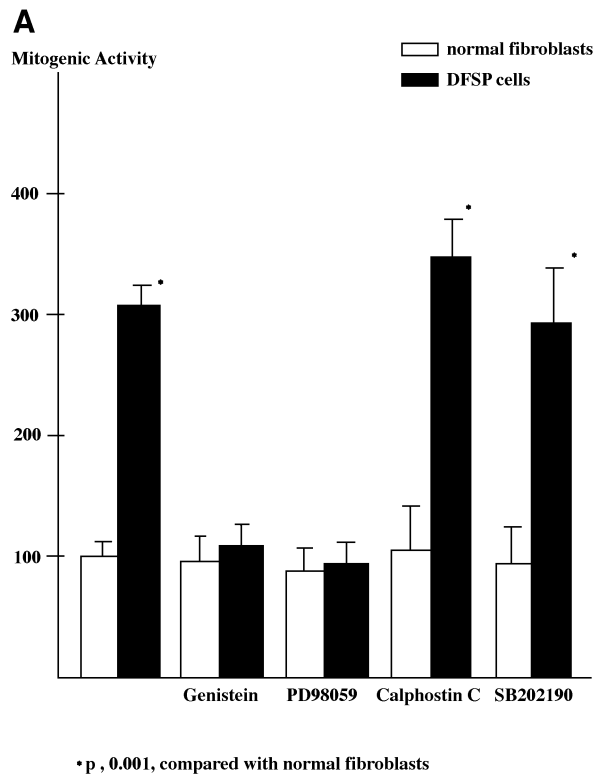
Statistical analysis Statistical analysis was carried out with the Mann–Whitney test for the comparison of means. p-values less than 0.05 were considered significant.

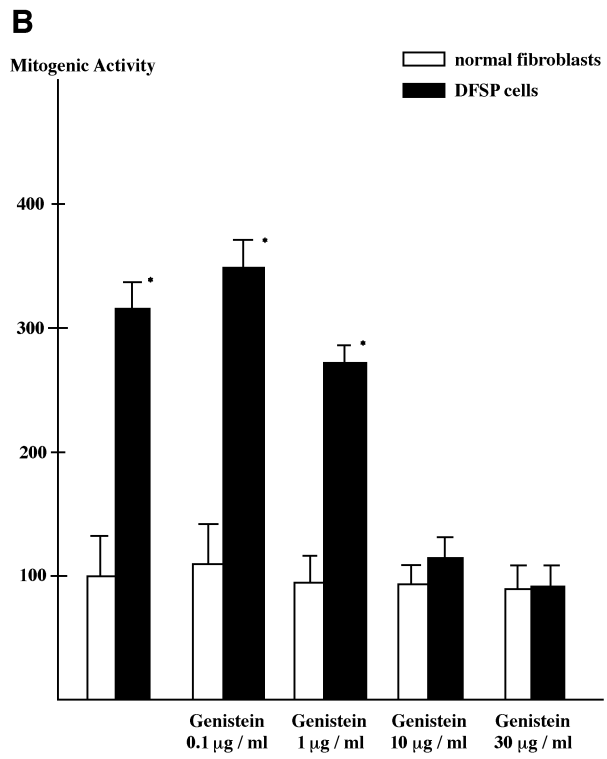
RESULTS

DFSP cells showed increased mitogenic activity To determine whether DFSP cells have increased mitogenic activity compared with normal dermal fibroblasts, we measured the incorporation of [^{3}H]-thymidine by DFSP cells and normal dermal fibroblast cultures in the absence of serum. The mean [^{3}H]-thymidine incorporation in serum-free MEM for each dermal fibroblast strain was arbitrarily set at 100%, and the [^{3}H]-thymidine incorporation for DFSP cells was estimated as a percentage ratio in each experiment. As shown in **Fig 1(*A*)**, DFSP cells showed increased mitogenic activity. The mitogenic activity of DFSP cells showed 200%–300% increases in DNA synthesis compared with normal dermal fibroblasts.

The effect of a tyrosine kinase inhibitor on the increased DNA synthesis of DFSP cells Previous studies reported that PDGF signaling involved tyrosine phosphorylation in various types of cells (Heldin *et al*, 1998). Therefore, we investigated the effects of a tyrosine kinase inhibitor on the increased DNA synthesis of DFSP cells. DFSP cells and normal dermal fibroblasts were treated with a tyrosine kinase inhibitor, genistein. Pretreatment of DFSP cells with genistein (30 μg per ml for 1 h) markedly decreased [^{3}H]-thymidine incorporation (**Fig 1*A***). Pretreatment of normal dermal fibroblasts with genistein also decreased [^{3}H]-thymidine incorporation by almost 60%. Furthermore, pretreatment of fibroblasts with PD98059, a specific ERK pathway inhibitor (30 μM for 1 h), inhibited increased [^{3}H]-thymidine incorporation of DFSP cells (**Fig 1*A***). Pretreatment of normal dermal fibroblasts with PD98059 also decreased [^{3}H]-thymidine incorporation. On the other hand, pretreatment with calphostin C, a protein kinase C inhibitor (5 μM), or SB202190, a p38 MAPK inhibitor (10 μM), had no effect on the increased mitogenic activity of DFSP cells or [^{3}H]-thymidine incorporation of normal dermal fibroblasts (**Fig 1*A***). Furthermore, pretreatment of DFSP cells with genistein (**Fig 1*B***) or PD98059 (**Fig 1*C***) inhibited increased mitogenic activity of DFSP cells in a dose-dependent manner. These results suggest that increased DNA synthesis of DFSP cells is mediated via an ERK-dependent pathway.

Constitutive nuclear localization of ERK in DFSP cells The localization of ERK was investigated using immunofluorescence analysis wih serum-deprived DFSP cells and normal human dermal fibroblasts. As shown in **Fig 2(*A*)**, ERK was





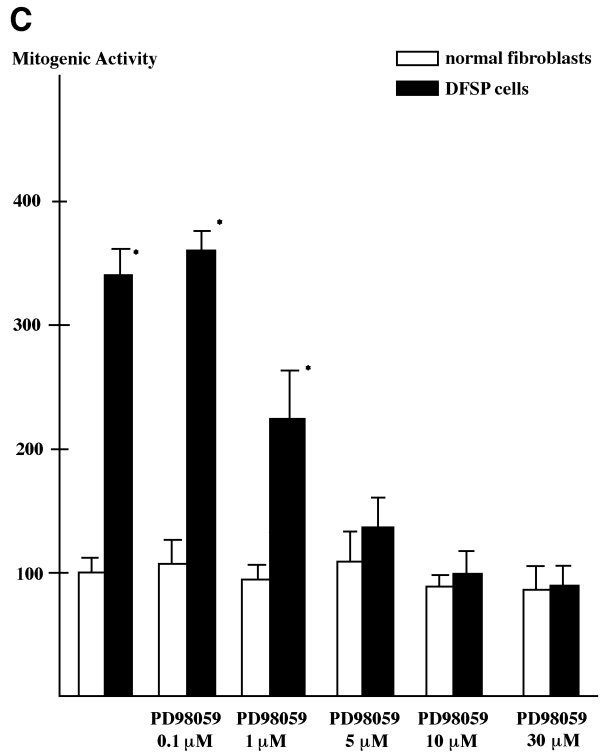


Figure 1. Mitogenic activity of DFSP cells. (*A*) DFSP cells showed increased mitogenic activity. The incorporation of [^{3}H]-thymidine by DFSP cells and normal dermal fibroblast cultures in the absence of serum was measured. The mean [^{3}H]-thymidine incorporation (cpm) in serum-free MEM for each dermal fibroblast strain was arbitrarily set at 100%, and the [^{3}H]-thymidine incorporation for DFSP cells was estimated as a percentage ratio in each experiment. The effects of genistein (30 μg per ml), PD98059 (30 μM), calphostin C (5 μM), or SB202190 (10 μM) were estimated as a percentage ratio. Pretreatment of DFSP cells with genistein markedly decreased the increased [^{3}H]-thymidine incorporation in DFSP cells. Furthermore, pretreatment of DFSP cells with PD98059 inhibited the increased [^{3}H]-thymidine incorporation in DFSP cells. Pretreatment with calphostin C or SB202190, however, had no effect on the increased mitogenic activity of DFSP cells. (*B*) Pretreatment of DFSP cells with genistein (0.1–30 μg per ml) decreased the increased [^{3}H]-thymidine incorporation in DFSP cells in a dose-dependent manner. (*C*) Pretreatment of DFSP cells with PD98059 (0.1–30 μM) decreased the increased [^{3}H]-thymidine incorporation in DFSP cells in a dose-dependent manner. The means ± SD for four separate experiments are shown. Comparisons of the [^{3}H]-thymidine incorporation (cpm) were made between DFSP cells and normal dermal fibroblasts (*$p < 0.01$).

located in the cytoplasm of normal fibroblasts without stimulation. ERK was constitutively located in the nucleus in DFSP cells, however (**Fig 2*C***). ERK blocking peptide was used to preabsorb the antibody, which indicated that the staining was specific (**Fig 2*E***).

Constitutive tyrosine phosphorylation of MAPK in DFSP cells The constitutive phosphorylation of ERK in DFSP cells was investigated using serum-deprived DFSP cells and normal human dermal fibroblasts. Detection of the phosphorylated forms of ERK was performed with antibodies specific for the phosphorylated sites (corresponding to Thr^{202}/Tyr^{204}) of the p44 and p42 ERK. Immunoblotting of whole cell extracts detected that constitutive ERK Thr^{202}/Tyr^{204} phosphorylation occurred in DFSP cells (**Fig 3*A***). Slight phosphorylation of ERK was also detected in some normal dermal firoblasts (**Fig 3*A***, *lanes 1*, *2*). Antibodies against ERK were also used to determine the protein concentrations of ERK in DFSP cells and

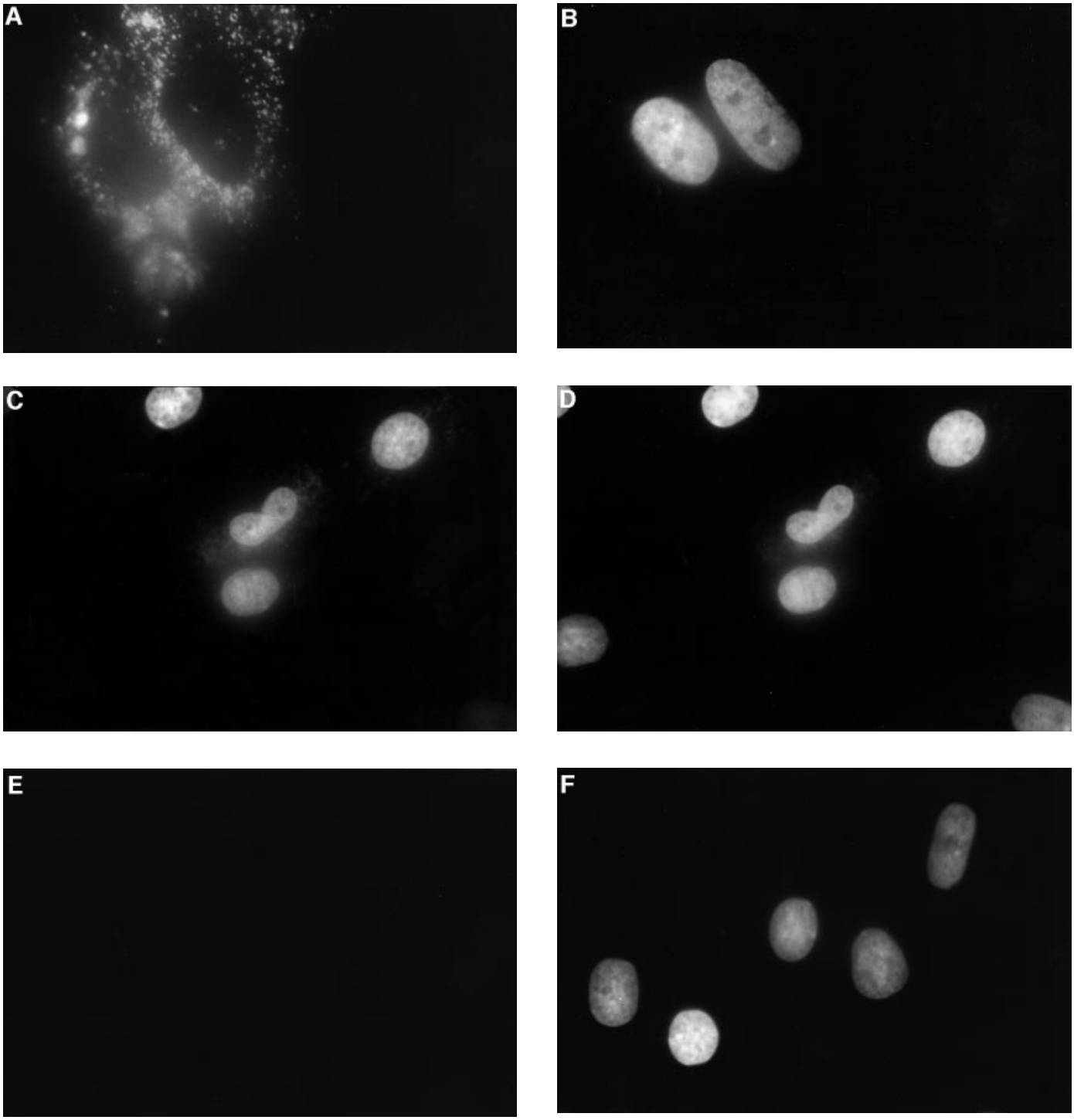


Figure 2. Constitutive nuclear localization of ERK in DFSP cells. The localization of ERK was investigated with immunofluorescence analysis using serum-deprived DFSP cells and normal human dermal fibroblasts as described in *Materials and Methods*. ERK was located in the cytoplasm of normal fibroblasts without stimulation (*A*). ERK was constitutively located in the nucleus in DFSP cells, however (*C*). ERK blocking peptide was used to preabsorb the antibody (*E*). Cells were counterstained with 4,6-diamidino-2-phenylindole to visualize nuclei (*B*, *D*, *F*).

normal dermal fibroblasts. Immunoblotting of whole cell extracts demonstrated that DFSP cells and normal dermal fibroblasts had an equivalent amount of ERK (**Fig 3*A***). We next examined whether ERK phosphorylation detected in DFSP cells was associated with increased ERK activity using an immunocomplex kinase assay. Increased ERK activity was also detected in DFSP cells (**Fig 3*B***).

The expression of the dominant negative mutant MAPK represses the increased DNA synthesis of DFSP cells To further confirm the role of MAPK in the growth regulation of DFSP cells, transient transfection of the dominant negative mutant ERK into DFSP cells and normal dermal fibroblasts was performed. As shown in **Fig 4**, transient transfection of the dominant negative mutant ERK in normal dermal fibroblasts decreased their [^{3}H]-

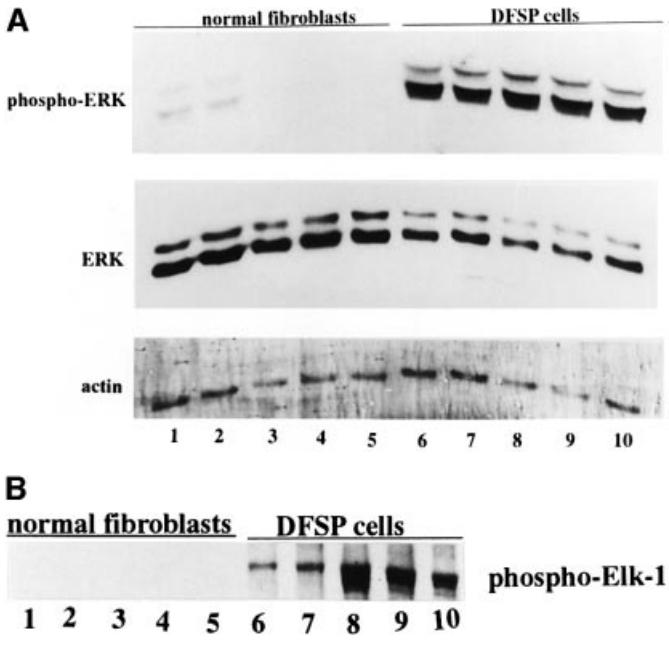


Figure 3. Constitutive tyrosine phosphorylation of MAPK in DFSP cells. (*A*) The constitutive phosphorylation of ERK in DFSP cells was investigated using serum-deprived DFSP cells and normal human dermal fibroblasts. Detection of the phosphorylated forms of ERK was performed with the antibody specific for the phosphorylated site (corresponding to Thr^{202}/Tyr^{204}) of the p44 and p42 ERK as described in *Materials and Methods*. Immunoblotting of whole cell extracts demonstrated that constitutive ERK Thr^{202}/Tyr^{204} phosphorylation occurred in DFSP cells. Antibodies against ERK were also used to determine the protein concentrations of ERK in DFSP cells and normal dermal fibroblasts, which demonstrated that DFSP cells and normal dermal fibroblasts have equivalent amounts of p44 and p42 ERK. Protein loading was confirmed using monoclonal anti-β-actin antibodies. (*B*) Immunocomplex kinase assay. Whole cell lysates were analyzed for ERK activity as described in *Materials and Methods*. ERK was assayed by immunoprecipitation with phospho-specific antibody to ERK followed by detection of phosphorylation of Elk-1 fusion protein at Ser^{383} by immunoblotting using a phospho-specific Elk-1 antibody.

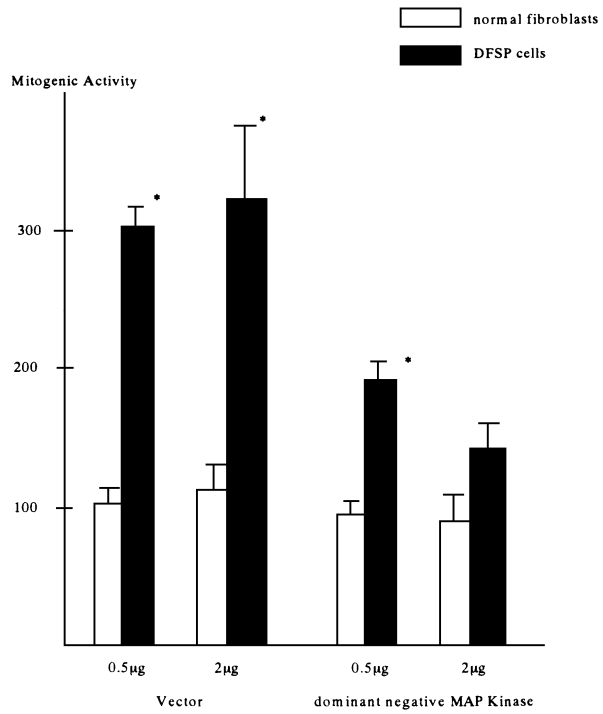


Figure 4. The expression of the dominant negative mutant ERK repressed the increased [^{3}H]-thymidine incorporation of DFSP cells. Transient transfection of the dominant negative mutant ERK or the empty vector into human dermal fibroblasts as described in *Materials and Methods*. The mean [^{3}H]-thymidine incorporation (cpm) in serum-free medium for each dermal fibroblast strain transfected with 0.5 μg of the empty vector was arbitrarily set at 100%, and the effects of the transfection of the dominant negative mutant ERK into DFSP cells and normal dermal fibroblasts were estimated as a percentage ratio at each concentration. The means ± SD for four separate experiments are shown. Comparisons of the [^{3}H]-thymidine incorporation (cpm) were made between DFSP cells and normal dermal fibroblasts (*$p < 0.01$).

thymidine incorporation by almost 50% in a dose-dependent manner. Furthermore, transient transfection of the dominant negative mutant ERK in DFSP cells also abolished increased [^{3}H]-thymidine incorporation of DFSP cells.

DISCUSSION

PDGF is a potent stimulator of growth and motility of connective tissue cells, such as fibroblasts and smooth muscle cells (Ross *et al*, 1986). It is a dimeric molecule consisting of disulfide-bonded A- and B-polypeptide chains. Homodimeric (PDGF-AA, PDGF-BB) as well as heterodimeric (PDGF-AB) isoforms exert their effects on target cells by binding with different specificities to two structurally related protein tyrosine kinase receptors, denoted the α- and β-receptors (Hart *et al*, 1988; Heldin *et al*, 1988). PDGF-AA induces αα receptor dimers, PDGF-AB αα or αβ receptor dimers, and PDGF-BB all three possible combinations of receptor dimers (Bishayee *et al*, 1989; Heldin *et al*, 1989; Seifert *et al*, 1989).

The intracellular transduction of signals involves direct interactions between components in the different signaling pathways. Such interactions are exerted by specific domains, such as Src homology 2 domains and phosphotyrosine binding domains, which recognize phosphorylated tyrosine residues in specific environments, SH3 domains, which recognize proline-rich regions, and pleckstin homology domains, which recognize membrane phospholipids (Schlessinger, 1993; Pawson and Scott, 1997). More than 10 different SH2-domain-containing molecules have been shown to bind to different autophosphorylation sites in the PDGF α- and β-receptors, including signal transduction molecules with enzymatic activity such as phosphatidylinositol 3′ kinase (PI3-kinase), phospholipase C-γ (PLC-γ), the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2, GTPase activating protein (GAP) for Ras, as well as adaptor molecules such as Grb2, Shc, Nck, Grb 7, and Crk, and signal transduction and activators of transcription (Stats) (Heldin *et al*, 1998). Activation of Ras by the PDGF β-receptor occurs by direct binding of Grb2/Sos1 to the phosphorylated Tyr^{716} of the receptor, or binding of Grb2/Sos1 to phosphorylated molecules bound to the receptor, like Shc or SHP-2 (Schlessinger, 1993; Arvidsson *et al*, 1994; Benjamin and Jones, 1994; Li *et al*, 1994; Yokote *et al*, 1994). Ras activation, leading to activation of the MAP kinase cascade, has been shown to be of major importance for growth stimulation, and overactivity of different components along this pathway has been shown to lead to cell transformation (Seger and Krebs, 1995).

This study demonstrated that DFSP cells showed increased mitogenic activity (**Fig 1*A***). This result is consistent with a previous report that showed that NIH 3T3 cells transfected with DFSP DNAs showed increased mitogenic activity (Greco *et al*, 1998). It was also reported that NIH 3T3 cells transfected with DFSP DNAs constitutively expressed the tyrosine-phosphorylated PDGF β-receptor (Greco *et al*, 1998). Little about the mechanism of the increased growth activity of DFSP cells has been clarified, however. In this study, increased DNA synthesis of DFSP cells was

significantly diminshed by a tyrosine kinase inhibitor, genistein, in a dose-dependent manner (**Fig 1*B***). This result suggests that phosphorylation of tyrosine kinase is involved in increased mitogenic activity of DFSP cells.

Next, we determined whether an ERK pathway was involved in the increased mitogenic activity of DFSP cells, using two independent approaches to block the ERK signaling pathway. First, we utilized an MEK1-specific inhibitor, PD98059, which blocks MEK1 activation by Raf, thus preventing downstream activation of ERK, but does not inhibit JNK or p38 MAPK. In addition, PD98059 has been shown to have little effect on other kinases, including cAMP-dependent kinase and protein kinase C (Dudley *et al*, 1995; Pang *et al*, 1995). In our study, pretreatment of DFSP cells with PD98059 inhibited their increased [^{3}H]-thymidine incorporation (**Fig 1*C***). Furthermore, transfection with the dominant negative mutant ERK into DFSP cells abolished their increased mitogenic activity (**Fig 4**).

MAPK modules are involved in the signal transduction of a wide variety of signals in all eukaryotic organisms. In mammalian cells, three well-characterized modules coexist: p44/p42 ERK, p38 MAPK, and JNK cascades (Cano and Mahadevan, 1995). The p42/p44 ERK cascade plays a pivotal role in the re-entry of fibroblasts into the cell cycle (Pages *et al*, 1993). Both p42 and p44 ERK are activated by dual phosphorylation on threonine and tyrosine residues, achieved via the dual-specificity kinase MAP kinase kinases (MEK) 1/2. Whereas MEK 1/2 remain permanently in the cytoplasm, p42/p44 ERK are relocalized from the cytoplasm to the nucleus upon stimulation (Chen *et al*, 1992). In fibroblasts, a correlation exists between the mitogenic potency of a stimulus and its ability to trigger p42/p44 ERK translocation (Chen *et al*, 1992). In this study, immunofluorescent analysis revealed that ERK was constitutively located in the nucleus in DFSP cells, although it was located in the cytoplasm of normal fibroblasts without stimulation (**Fig 2**). Moreover, immunoblotting analyses using phospho-specific ERK antibodies that detect only the Thr^{202}/Tyr^{204}-phosphorylated forms of ERK1/ERK2 demonstrated that constitutive ERK Thr^{202}/Tyr^{204} phosphorylation was detected in DFSP cells (**Fig 3**). These results suggest that constitutive ERK Thr^{202}/Tyr^{204} phosphorylation make continual ERK translocation from the cytoplasm to the nucleus. SB202190, however, a p38 MAPK inhibitor, had no effect on the increased mitogenic activity of DFSP cells or [^{3}H]-thymidine incorporation of normal dermal fibroblasts (**Fig 1*A***). Furthermore, increased phosphorylation of JNK was not detected in DFSP cells (data not shown). These results suggested that neither p38 MAPK nor JNK is involved in increased mitogenic activity of DFSP cells.

The ERK pathway is the prototypical MAPK pathway induced by other growth factor stimulation and it is implicated in the regulation processes of cellular proliferation (Blumer and Johnson, 1994; Kyriakis *et al*, 1994). A recent study demonstrated that ERK was heavily phosphorylated on tyrosyl residues and its activity was elevated in human breast cancer compared to benign conditions (Sivaraman *et al*, 1997).

It will be of great interest to determine whether similar activation of ERK-dependent signaling pathways exists in other human neoplasia, and our findings may potentially have important clinical implications in DFSP.

Furthermore, the downstream pathways of ERK, such as ELK-1, Sap-1, c-Myc, $p90^{rsk}$, MAPKAPK2, MNK1, and MNK2, have been reported and some of them may influence mitogenic activity (Frost *et al*, 1997; Rausch and Marshall, 1999; Sears *et al*, 1999; Knauf *et al*, 2001; Noguchi *et al*, 2001). Further studies are needed to clarify the involvement of these kinases in increased mitogenic activity of DFSP cells.

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