***Brief Definitive Report***

# IDENTIFICATION OF THE HUMAN 26-kD PROTEIN, INTERFERON $\beta_2$ (IFN-$\beta_2$), AS A B CELL HYBRIDOMA/PLASMACYTOMA GROWTH FACTOR INDUCED BY INTERLEUKIN 1 AND TUMOR NECROSIS FACTOR

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Recently, mouse B cell hybridomas and plasmacytomas have been described (1, 2) whose in vitro growth is strictly dependent on a mouse T cell–derived factor provisionally designated interleukin-HP1 (IL-HP1). This 22–29 kD protein, which was purified to homogeneity and partially sequenced, shows no homology with known cytokines. Its relation to the P388-D1 macrophage line–derived plasmacytoma growth factor (3) has not yet been defined. We have also observed that the supernatant of human fibroblast cultures stimulated by double-stranded RNA or IL-1 can sustain the growth of the IL-HP1-dependent hybridoma cell lines (4). Both stimuli for this hybridoma growth factor (HGF) activity are also known to be inducers of IFN-$\beta_1$ (5). However, since pure IFN-$\beta_1$ was devoid of HGF activity (4), we hypothesized that a substance coinduced with IFN-$\beta_1$ is responsible for the HGF activity. One such substance is a protein termed 26 kD by some workers (6, 7) and IFN-$\beta_2$ by others (8). Its IFN-like antiviral activity, which has formed the basis for its denomination as IFN-$\beta_2$, is rather weak, and this has raised the question of a possible other biological function for this protein.

## Materials and Methods

*Cell Cultures.* B cell hybridomas were 6H8 (rat-mouse) and 7TD1 (mouse-mouse), both dependent for their growth on the T cell–derived HGF, IL-HP1 (1), IL-HP1-dependent mouse plasmacytoma lines were derived from TEPC 1033, MOPC 104E, MOPC 173, and RPC 20, as described (2).

*Assay Systems.* Antiviral activity of cytokines is expressed in antiviral units/ml (AVU/ml) as determined on $E_6$SM diploid fibroblasts or MG-63 osteosarcoma cells using

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a cytopathic effect reduction assay with vesicular stomatitis virus as a challenge virus (4). The assay for HGF was performed by incubating 7TD1 or 6H8 cells with serial dilutions of growth factor and by colorimetric determination of cell numbers (1). The plasmacytoma growth activity was determined by measuring [$^3$H]thymidine incorporation (2).

*Cytokines.* Natural IL-1$\beta$ (22 kD factor) was obtained from Con A–stimulated PBL and purified to homogeneity as described (9); recombinant DNA–derived IL-1$\alpha$, IL-1$\beta$, and TNF-$\alpha$, produced in *Escherichia coli*, were obtained by courtesy of Prof. C. Dinarello, Tufts University School of Medicine, Boston, MA (origin of IL-1$\alpha$, Genzyme Inc., Boston, MA; origin of TNF-$\alpha$, Genentech, San Francisco, CA).

*Production of HGF.* HGF was produced in MG-63 cells, induced with IL-1$\beta$. The supernatant fluid was processed through a five-step concentration and purification schedule as follows: (*a*) batch adsorption to controlled pore glass (CPG) and desorption by acid; (*b*) immuno-affinity chromatography using a polyclonal antibody against CPG-purified HGF; (*c*) gel filtration on Ultro-gel AcA54 (LKB Produkter, Bromma, Sweden); (*d*) FPLC on a cation exchange column (Mono S, Pharmacia Fine Chemicals, Uppsala, Sweden); (*e*) reversed-phase HPLC on a $C_1$ 250 Å pore size TSK TMS-250 column (LKB Produkter). $NH_2$-terminal sequence analysis was carried out on a gas phase sequencer (model 478; Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin analyzer (model 120A; Applied Biosystems, Inc.).

*RNA-DNA Hybridization.* DNA from the following plasmids were used: pBR322; p26K-1, a pBR322 derivative containing a 737-bp cDNA sequence for 26K, originally isolated by Haegeman et al. (7); and pAB48K-5, a pBR322 derivative containing a 1,021-bp cDNA sequence for a human elongation factor 1$\alpha$. 10 $\mu$g of CsCl-purified plasmid DNA was denatured and applied to 1-cm$^2$ nitrocellulose filters. After baking and washing, the filters were shaken (3 h, 47°C) in hybridization buffer (500 $\mu$l) containing 40 $\mu$g of RNA. Residual unbound RNA (R), as well as RNA in two 500 $\mu$l washings ($W_1$ and $W_2$; 10 min, 47°C) were collected. The plasmid-bound RNA was then eluted from the filters by heating (1 min, 100°C) in 300 $\mu$l distilled water containing 20 $\mu$g/ml *E. coli* tRNA. The eluate (E) was also collected. A fixed fraction of all collected fluids was used directly for RNA/dot-blot assays against the 26K cDNA probe (a 480-bp Sau 3A cDNA fragment of 26K cDNA [7], sp act ~$1.9 \times 10^9$ cpm/$\mu$g); the remaining fraction was alcohol-precipitated. The pellets were washed twice in 75% cold ethanol, redissolved in 50 (R), 10 ($W_1$ and $W_2$), and 5 $\mu$l (E) bidistilled water and used for injection in *Xenopus* oocytes (50 nl per oocyte; 10 oocytes per RNA sample). After 48 h incubation culture fluids were collected, supplemented with 10% FCS, and assayed for HGF activity.

## Results and Discussion

Production of HGF by cells treated with various cytokines is documented by Table I. Cultures of human diploid fibroblasts or MG-63 osteosarcoma cells were treated with pure natural IL-1$\beta$ or rIL-1$\alpha$, rIL-1$\beta$, or rTNF-$\alpha$, given at increasing doses. Natural IL-1$\beta$ was able to induce production of HGF by both cell types. The minimum active doses of IL-1 required for significant induction were 0.1–1 AVU/ml, and maximum production levels amounted to $10^4$ to $10^{4.4}$ U/ml HGF activity. The HGF-inducing effect of natural IL-1$\beta$ was confirmed with rIL-1$\beta$. In addition, IL-1$\alpha$ was found to be equally potent and TNF-$\alpha$ less potent as inducers of HGF in diploid fibroblasts. Control experiments indicated that none of the cytokines had HGF activity by themselves.

A first type of evidence proving relatedness of IL-1-induced HGF to the 26 kD protein consisted in the demonstration that the mRNA for HGF binds to immobilized 26 kD cDNA. Poly(A)$^+$ RNA was extracted from MG-63 cell monolayers that had been incubated for 6 h with 200 AVU/ml of IL-1$\beta$. In four of five consecutive experiments, *Xenopus* oocytes injected with this RNA secreted

TABLE I

*Induction of HGF by Stimulation of Different Human Cell Lines by IL-1α, IL-1β, and TNF-α*

| Inducing cytokine‡ | Cell line | Yield of HGF ($log_{10}$ U/ml) after stimulation with cytokine dose* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 (control) | 0.1 | 1 | 10 | 100 | 1,000 |
| IL-1β (N) | $E_6$SM | 1.40 (± 0.33)§ | 2.10 (± 0.45) | 3.17 (± 0.19) | 3.87 (± 0.08) | 4.40 (± 0.21) | — |
| | MG-63 | 2.38 (± 0.11) | 2.76 (± 0.08) | 3.26 (± 0.31) | 3.81 (± 0.26) | 3.94 (± 0.18) | — |
| IL-1β (R) | $E_6$SM | 2.2 | 2.9 | 2.9 | 3.4 | 4.7 | — |
| IL-1α (R) | $E_6$SM | <2.0 | — | 2.7 | 3.6 | 4.4 | — |
| TNF-α (R) | $E_6$SM | <2.0 | — | 2.6 | 2.8 | 3.3 | 4.0 |

Confluent cell cultures (1.9 $cm^2$; 0.5 ml) were incubated at 37°C for 2 h with medium (+2% FCS) containing IL-1 or TNF-α, using duplicate sets for each indicated dose. The cultures were washed, *replenished* with plain medium, and further incubated for 24 h. Supernatant medium was then harvested for HGF determination.

* Doses of cytokine are given in AVU/ml as assayed by antiviral effect on MG-63 cells; 1 U equals ~1 ng.

‡ N, natural; R, recombinant.

§ Mean yield over 5 experiments; in parentheses: standard error.

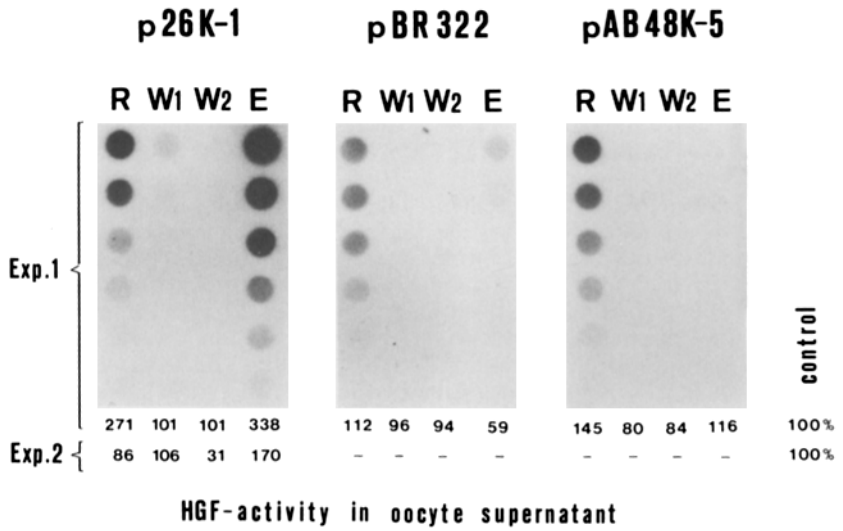


FIGURE 1. Identification of 26 kD mRNA as HGF mRNA by hybrid-selected translation. Poly(A)-rich RNA samples containing mRNA for HGF were obtained (5) from MG-63 cells incubated for 6 h with 200 AVU/ml of IL-1β. The samples were processed for hybrid selection on three different filter-immobilized plasmid DNAs: p26K-1, pBR322, and pAB48K-5. After hybridization of the RNA to the filters, residual unbound RNA (R), as well as RNA in two washings ($W_1$ and $W_2$) were collected. The plasmid-bound RNA was then eluted (E). The four fractions (R, $W_1$, $W_2$, and E) were then tested for the presence of HGF mRNA content by translation in oocytes. In Exp. 1 the RNA fractions were also tested for the presence of 26 kD cDNA sequence by dot-blot assay, using one-third dilution steps. HGF activity in the oocyte supernatant is given as cell outgrowth numbers in percent of control cultures without HGF. Each data set from Exp. 1 represents averages of two oocyte injection experiments. The HGF activities from Exp. 2 are averages from three oocyte injection experiments.

on average 30 U/ml/48 h of HGF activity against undetectable amounts in controls receiving no RNA or RNA from untreated MG-63 cells. Samples of IL-1-induced HGF mRNA were processed for hybridization to cloned 26 kD cDNA. The results of these experiments are illustrated in Fig. 1, which shows analysis of the RNA after enrichment on a 26 kD cDNA, both by dot-blot detection with a 26 kD cDNA probe and by oocyte translation into HGF. Enrichment in 26 kD mRNA sequences is clear from the comparison of the dot-blot pattern obtained

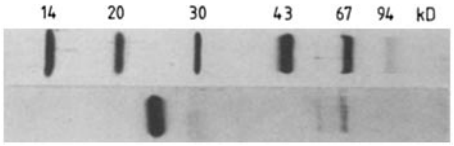


FIGURE 2. SDS-PAGE of purified fibroblast-derived HPGF. A sample (2 μl) of the peak activity from reversed-phase HPLC was run on SDS-PAGE (15% gel) under reducing conditions and was silver stained. The two high molecular mass bands in the test sample (*bottom*) are staining artefacts.

TABLE II

*Growth-Promoting Activity of Fibroblast-derived HGF for Plasmacytoma Cell Lines*

| Test material | Dilution ($-\log_{10}$) | Plasmacytoma cell growth activity (cpm) | | | |
|---|---|---|---|---|---|
| | | TEPC 1033 | MOPC 104E | MOPC 173 | RPC 20 |
| Partially purified HGF (affinity chromatography) | 3.0 | 30,300 | 38,600 | 17,900 | 26,600 |
| | 3.7 | 3,300 | 4,500 | 4,100 | 1,000 |
| | 4.4 | 100 | 650 | 1,100 | 150 |
| | 5.1 | 100 | 250 | 100 | 100 |
| Control | | 100 | 200 | 450 | 100 |
| Homogeneous HGF (reversed-phase HPLC) | 3.0 | 26,933 ± 1182* | — | — | — |
| | 3.7 | 13,691 ± 1676 | — | — | — |
| | 4.4 | 217 ± 22 | — | — | — |
| | 5.1 | 54 ± 6 | — | — | — |
| Control | | 50 ± 6 | — | — | — |

* Mean ± SE; $n = 4$.

with RNA eluted from a 26 kD cDNA filter, with that obtained after elution from filters containing pBR322 plasmids without 26 kD sequences. Concomitant enrichment in HGF mRNA sequences is apparent from the fact that the RNA eluted from 26 kD cDNA contained oocyte-translatable HGF mRNA while similar RNA eluted from the filters carrying irrelevant DNAs scored negative in the HGF assay. This concomitant adsorption to immobilized 26 kD cDNA of 26 kD mRNA sequences and of HGF-translatable mRNA strongly supports the contention of close relatedness between the two molecules.

More compelling evidence was obtained by purification to homogeneity and sequence analysis of the naturally produced biologically active factor. The supernatant fluid of IL-1-induced MG-63 cells was collected and processed through a five-step concentration and purification schedule (see Materials and Methods). The end product was shown to be electrophoretically homogeneous as illustrated in Fig. 2. The molecular mass of the pure protein was estimated at 24 kD and biological activity was eluted from gels at the same molecular mass position. The pure protein (about 10 μg) was used for amino-acid sequence analysis and the $NH_2$-terminal sequence was found to be as follows: ALA-PRO-VAL-PRO-PRO-GLY-GLU-ASP-SER-LYS-ASP-VAL-. . . This sequence exactly matches that predicted from the nucleic acid sequence of the 26 kD cDNA (7, 8). The latter analysis also identifies the cleavage site between the signal peptide (27 amino acids) and the mature protein.

That the human fibroblast–derived HGF could also act as a growth factor for factor-dependent mouse plasmacytoma cell lines is documented by Table II.

Partially as well as completely purified HGF supported the growth of several of those cell lines in a dose-dependent fashion.

It can be concluded that the IL-1-induced human fibroblast–derived hybridoma/plasmacytoma growth factor (HPGF) is identifiable as the 26 kD/IFN-$\beta_2$ protein with the possibility of some differences outside the $NH_2$-terminal portion of the molecule. Unlike IFN-$\beta_1$, the factor crosses the species barrier from man to mouse, a phenomenon seen with various other cytokines (e.g., IL-1 and IL-2). Furthermore, the factor is serologically related (4) to similar growth factors from human leukocytes (1, 10). The portion of the $NH_2$-terminal amino acid sequence that is presently known (1) for the mouse T cell–derived HPGF (IL-HP1) shows no significant degree of homology with the human fibroblast–derived factor. However, this does not rule out the existence of homologous regions elsewhere in the molecule.

With respect to the physiological target of HPGF it should be mentioned that all hybridoma and plasmacytoma growth factors discovered so far, seem to be different from the classical B cell growth and differentiation factors, BSF-1 and BCGF-II (1, 3). This was also the case for the HPGF described here, which, in spite of its activity on murine plasmacytomas, had no effect in a murine BSF-1 assay.

The natural inducers of HPGF known so far are IL-1 and TNF-$\alpha$. Both these molecules have an antiviral effect on cells (5, 11), and this effect has been postulated to be due to induction of either IFN-$\beta_1$ (5) or 26 kD/IFN-$\beta_2$ (11). Homogeneous preparations of natural HPGF ($50 \times 10^6$ HGF U/ml) did not show significant antiviral activity (<30 AVU/ml) in any of our assays in which IL-1 or TNF-$\alpha$ were antivirally active. The specific antiviral activity of HPGF was $<10^4$ AVU/mg, allowing us to state that if it were to be the mediator of the antiviral effect of IL-1 or TNF, at least 10,000-fold more of it would need to be produced than if IFN-$\beta_1$ ($\geq 10^8$ AVU/mg) would be the mediator.

After submission of the present report, Hirano et al. (12) have published the amino acid sequence of BSF-2. This sequence is identical to that of the 26 kD protein, indicating that BSF-2 is also a growth factor.

## Summary

A factor that promotes the growth of certain B cell hybridomas and of plasmacytomas is shown to be produced by normal human fibroblasts and by a line of human osteosarcoma cells (MG-63) after treatment with IL-1 or TNF. The hybridoma-plasmacytoma growth factor (HPGF) is identified with a 26 kD protein whose mRNA was previously shown to be induced in the same cells by the same inducers. First, poly(A)-rich RNA extracted from IL-1-treated cells could be enriched in HPGF-mRNA content by hybridization to 26 kD cDNA. Second, MG-63-derived HPGF purified to electrophoretic homogeneity was subjected to amino acid sequence analysis, whereby the $NH_2$-terminal sequence was found to match the nucleotide sequence of a 26 kD cDNA clone.

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