INHIBITION OF PROTEIN BREAKDOWN IN CULTURED CELLS IS A CONSISTENT RESPONSE TO GROWTH FACTORS

F. J. BALLARD, S. E. KNOWLES, Susanna S. C. WONG, J. B. BODNER*, Carla M. WOOD* and J. M. GUNN*
CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide, SA 5000, Australia and **Department of Biochemistry and Biophysics, Texas A & M University, College Station, TX 77843, USA*

Received 22 February 1980

1. Introduction

A number of small proteins have been purified and shown to partially replace the serum requirement for the growth of cells in culture [1–3]. Generally the response to these growth factors has been quantified as either a stimulus of DNA synthesis or an increase in cell number [1–7]. Although protein synthesis is stimulated, significant increases are not readily measured until several hours after addition of the growth factor [4]. Since a decrease in the rate of intracellular protein degradation can contribute to protein accumulation equally well as a stimulus in protein synthesis, we have examined rates of protein breakdown after the addition of purified growth factors to various cell lines. The experiments show a consistent and marked inhibition of protein degradation of physiological concentrations of insulin, EGF, FGF, IGF-I and IGF-II in cells known to be responsive to these factors.

2. Materials and methods

2.1. *Materials*

Fetal calf serum was obtained from Commonwealth Serum Laboratories, Melbourne (lot 171-2); insulin (Actrapid) from Novo Industri, Copenhagen; EGF and FGF from Collaborative Research, Waltham, MA; IGF-I and IGF-II from Dr R. E. Humbel, University of Zurich. L-[4,5-^{3}H]leucine (40–60 Ci/mmol) was from either New England Nuclear or The Radiochemical Centre, Amersham. Sources of culture media have been described in [8].

Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF-I, IGF-II, insulin-like growth factors I and II

2.2. *Cell cultures*

The sources of the different cell lines were: MH_1C_1 hepatoma, Swiss 3T3 fibroblasts and 3T12-3 fibroblasts, American Type Culture Collection, Rockville, MD; IMR-90 human fibroblasts and AG-2804 transformed human fibroblasts, Institute for Medical Research, Camden, NJ; Balb/c 3T3 and SV40-transformed Balb/c 3T3 fibroblasts, Dr Stuart Aaronson, National Cancer Institute, Bethesda, MD; HTC hepatoma, Dr W. D. Wicks, Department of Pharmacology, University of Colorado, CO; H35 hepatoma, Dr Joyce Becker, University of Wisconsin, Madison, WI; BEN bronchial carcinoma, rat osteogenic sarcoma and newborn rat calvarial osteoblasts, Drs T. J. Martin and N. C. Partridge, Repatriation General Hospital, West Heidelberg, VIC 3077; NRK wild type and NRK-442, Dr E. M. Scolnick, National Cancer Institute, Bethesda, MD. Primary cultures of adult rat hepatocytes were prepared from tissue perfused with collagenase [9]; adipose fibroblasts were from rat epididymal tissue [10] and chick embryo line fibroblasts from 9-day embryos [11]. The liver line was an epithelial-like cell line derived from a monolayer of parenchymal cells which had been prepared from a 10-day-old rat by collagenase/trypsin digestion.

2.3. *Measurement of protein degradation*

Confluent cell monolayers in Costar 24-well dishes were incubated for 16 h in Dulbecco-modified minimal essential medium containing 10% fetal calf serum, penicillin G (60 mg/l), streptomycin sulphate (100

mg/l), gentamycin (50 mg/l) and 1 μCi [^{3}H]leucine/ml with unlabelled leucine omitted. This labelling medium was removed, the monolayers washed twice with 'degradation medium' (minimal essential medium containing the antibiotics listed above, 2 mM leucine, 0.1% bovine serum albumin and 20 mM *N*-Tris[hydroxymethyl] methyl-2-aminoethane sulphonic acid) and a further 1 ml of this medium added to each well and left for 3 h to permit the breakdown of unstable proteins. Subsequently this medium was replaced with 1 ml of similar medium with serum or growth factors added at the concentrations indicated. After 4 h portions of the medium were taken for the determination of trichloroacetic acid-soluble and -insoluble radioactivity and the monolayer was dissolved in 1 ml

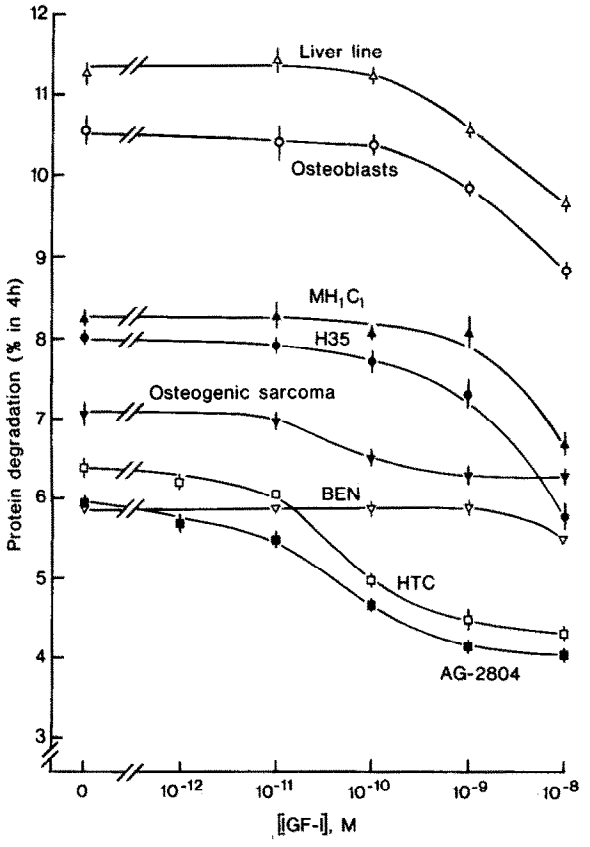

Fig.1. Concentration dependence of IGF-I inhibition of protein degradation. Values are means ± SEM for 6 determinations at each IGF-I concentration. The cell lines used were calvarial osteoblast-like cells (○), a liver line (△), MH$_1$C$_1$ (▲), H35 (●), osteogenic sarcoma (▼), HTC (□), AG-2804 (■) and BEN (▽).

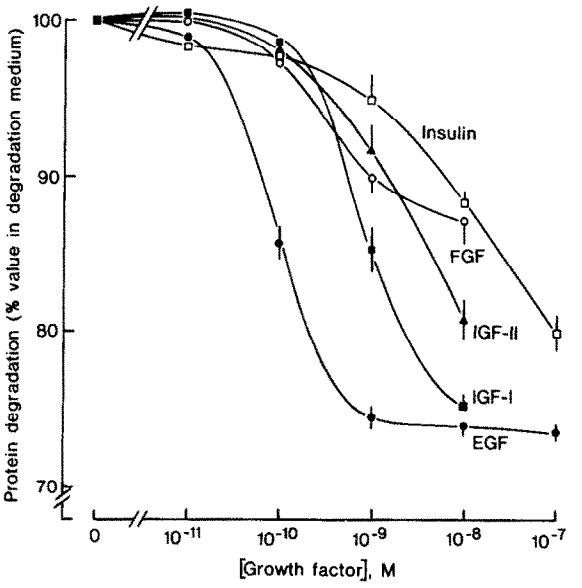

Fig.2. Inhibition of protein degradation produced by growth factors added to IMR-90 cells. The growth factors used were insulin (□), FGF (○), IGF-I (■), IGF-II (▲) and EGF (●). Values are means ± SEM for 6–12 determinations at each concentration and are expressed as percentages of the degradation measured in 'degradation medium'.

0.1 N NaOH containing 0.5% Triton X100. The percent degradation is calculated at 100-times the radioactivity in the medium trichloroacetic acid-soluble fraction divided by the total radioactivity in each well. Appropriate zero time values (typically 0.1–0.2% degradation) are subtracted.

3. Results and discussion

Concentration effects of IGF-I on protein breakdown are shown in fig.1. Inhibition of the pathway by 10^{-8} M IGF-I occurred in all 8 of the cell lines tested, with effects ranging from a 5% (BEN carcinoma) to a 30% (AG-2804) reduction. The cells appear to fall into two groups when sensitivity of the growth factor is considered. Thus calvarial osteoblasts, the liver line, BEN bronchial carcinoma as well as the MH$_1$C$_1$ and H35 hepatomas are not affected at 10^{-10} M IGF-I but show inhibition at higher concentrations. A sensitivity comparable to this group of cell lines has been described for DNA synthesis in chick embryo fibroblasts [6]. The response with HTC hepatoma, AG-2804 transformed human fibroblasts and the

osteogenic sarcoma is displaced by ~2 orders of magnitude towards lower IGF-I concentrations. Half-maximal effects are found in these cells at $\sim 3 \times 10^{-11}$ M. This 'sensitive' group contains only transformed cells whereas the first group comprises both contact-inhibited and transformed cells. IGF-I has been reported present at 2×10^{-8} M in normal human serum [6], a concentration which would markedly inhibit protein degradation in all the cell lines shown in fig.1 except for the BEN carcinoma.

Mitogenic effects in fibroblasts have been described after addition of EGF [1,5], FGF [2,7], IGF-I and IGF-II [6] as well as high concentrations of insulin [7]. Measurements with IMR-90, a human diploid fibroblast line [12], show inhibition of protein breakdown by all these growth factors (fig.2). Half-maximal effects are seen at approx. 10^{-10} M EGF, 5×10^{-10} M FGF, 10^{-9} M IGF-I, 2×10^{-9} M IGF-II and 10^{-8} M insulin. The magnitude of the responses also differs, although our inability to test some of the growth factors at $>10^{-8}$ M precludes comparison of maximal effects. With the exception of studies showing inhibition of protein breakdown by insulin in several cell lines [8,9,13,14], the only experiments where purified growth factors have been tested on the pathway have been those in [15] where 16% inhibition by 10^{-8} M FGF in rat embryo fibroblasts was found. The response was potentiated when insulin and dexamethasone were added with FGF.

A summary of growth factor effects on protein degradation for 19 cell lines is given in table 1. For simplicity the data are shown as the percentage inhibition of degradation produced when the growth factor was added at 10^{-8} M. Effects with a single batch of fetal calf serum are listed for comparison for 12 of the lines. It is evident that serum, IGF-I and

Table 1
Inhibition of protein degradation by growth factors

Cell line	Percent inhibition of protein degradation produced by					
	Serum	Insulin	EGF	FGF	IGF-I	IGF-II
Contact-inhibited						
Hepatocytes	17	20	7		5	13
Liver line	30	<5	24		14	8
IMR-90	19	12	26	13	25	19
Balb/c 3T3		28	15	26		
Swiss 3T3		24	8	16		
Chick embryo fibroblasts		22	7	<5		
Adipose fibroblasts	24	9	7	<5		
NRK-wild type		8	13			
Calvarial osteoblasts	28	<5	5		16	12
Transformed						
H35	27	30	<5		29	33
HTC	35	27	<5		31	29
MH_1C_1	24	25	<5		20	23
tr-IMR	24	19	<5		38	32
AG-2804	34	22	8	<5	33	26
SV40-3T3		19	14	12		
3T12-3		16	10	11		
NRK-442		6	<5			
BEN	13	15	<5		5	9
Osteogenic sarcoma	13	6	5		10	10

Protein degradation was determined as in section 2. Data are expressed as percentage effects produced by the growth factors as compared to 'degradation medium'. Fetal calf serum was added to give a 20% (v/v) solution; insulin, EGF, FGF, IGF-I and IGF-II were all added at 10^{-8} M

IGF-II inhibited protein degradation in every cell line tested, while insulin effects were obtained in all cells except a liver line and calvarial osteoblasts. Our unpublished experiments have shown extremely low binding of ^{125}I-labelled insulin in these two lines. Significant inhibition of protein breakdown by EGF occurred in all the contact-inhibited lines with the largest responses occurring in IMR-90 fibroblasts, the liver line and Balb/c 3T3 cells. Smaller effects of EGF were noted in both cell lines that had been transformed with SV40 virus (AG-2804, SV40-3T3), 3T12-3 cells and the osteogenic sarcoma. However, none of the hepatomas showed a response to EGF. Protein breakdown was inhibited by FGF in three of the contact-inhibited fibroblasts and in SV40-3T3 and 3T12-3 mouse fibroblasts.

Any common mechanism accounting for the inhibition of protein breakdown by growth factors is probably dependent on their binding to surface receptors. Thus all cell lines tested in which protein breakdown is inhibited by EGF or insulin have high affinity receptors for these agents (F. J. B., S. E. K., J. B. B., J. M. G., S. S. C. W., N. C. Partridge, T. J. Martin, unpublished). The post-receptor mechanism for insulin action on protein breakdown apparently involves a reduction in the number and size of autophagic vacuoles [16–18]. Since we have no information on the proportion of lysosomes or autophagic vacuoles in cells exposed to growth factors other than insulin, an extension of this concept to all growth factors is perhaps premature. However, in measurements on Balb/c 3T3 and IMR-90 cells, the addition of either EGF or FGF together with concentrations of insulin that produced maximal inhibitions of protein breakdown gives no extra effect (S. E. K., F. J. B., J. B. B., and J. M. G., unpublished). This is consistent with all three factors acting via a common mechanism.

Acknowledgements

We thank Dr R. E. Humbel, University of Zurich, for generously providing IGF-I and IGF-II. This work was supported in part by USPHS grant AM19891.

References

[1] Cohen, S. (1962) J. Biol. Chem. 237, 1555–1562.
[2] Gospodarowicz, D. (1975) J. Biol. Chem. 25, 2515–2520.
[3] Rinderknecht, E. and Humbel, R. E. (1976) Proc. Natl. Acad. Sci. USA 73, 2365–2369.
[4] Rudland, P. S. and De Asua, L. J. (1979) Biochim. Biophys. Acta 560, 91–133.
[5] Carpenter, G. and Cohen, S. (1979) Annu. Rev. Biochem. 48, 193–216.
[6] Zapf, J., Rinderknecht, E., Humbel, R. E. and Froesch, E. R. (1978) Metabolism 27, 1803–1828.
[7] Gospodarowicz, D. and Moran, J. S. (1976) Annu. Rev. Biochem. 45, 531–558.
[8] Knowles, S. E. and Ballard, F. J. (1976) Biochem. J. 156, 609–617.
[9] Gunn, J. M., Clark, M. G., Knowles, S. E., Hopgood, M. F. and Ballard, F. J. (1977) Nature 266, 58–60.
[10] Björntorp, P., Karlsson, M., Pentoft, H., Pettersson, P., Sjöström, L. and Smith, U. (1978) J. Lipid Res. 19, 316–324.
[11] Todaro, G. J. and Green, H. (1963) J. Cell Biol. 17, 299–313.
[12] Nichols, W. W., Murphy, D. G., Cristofalo, V. J., Toji, L. H., Greene, A. E. and Dwight, S. A (1977) Science 196, 60–63.
[13] Hershko, A., Mamont, P., Shields, R. and Tomkins, G. M. (1971) Nature New Biol. 232, 206–211.
[14] Gelehrter, T. D. and Emanuel, J. R. (1974) Endocrinology 94, 676–684.
[15] Warburton, M. J. and Poole, B. (1977) Proc. Natl. Acad. Sci. USA 74, 2427–2431.
[16] Mortimore, G. E. and Mondon, C. E. (1970) J. Biol. Chem. 245, 2375–2383.
[17] Ward, D. F., Cox, J. R. and Mortimore, G. E. (1977) J. Biol. Chem. 252, 6955–6961.
[18] Pfeifer, U. (1977) Acta Biol. Med. Germ. 36, 1691–1694.