Insulin-like Growth Factor-binding Protein (IGFBP-3)
Predisposes Breast Cancer Cells to Programmed Cell Death in a Non-IGF-dependent Manner*

(Received for publication, May 7, 1997, and in revised form, July 23, 1997)

Zahidah P. Gill, Claire M. Perks, Paul V. Newcomb, and Jeff M. P. Holly‡
From the Division of Surgery, Department of Hospital Medicine, Bristol Royal Infirmary, Bristol, BS2 8HW, United Kingdom

Insulin-like growth factor (IGF)-independent growth inhibition of human breast cancer cells, Hs578T, by IGFBP-3 has been previously been demonstrated. Cell growth is a balance between proliferation and programmed cell death (apoptosis). We have investigated whether IGFBP-3 can affect apoptosis of Hs578T cells. As no induction of apoptosis was found, we also investigated its effect on the response to ceramide, an intracellular second messenger that mediates the signal for apoptosis. Using the cell permeable ceramide analogue, C2, induction of apoptosis was established by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, trypan blue uptake, morphological criteria, and flow cytometry. Incubation of cells with non-glycosylated IGFBP-3 (ngIGFBP-3; 0.5–100 ng/ml) resulted in no growth inhibition or increase in apoptosis; whereas, C2 (1–30 μM) resulted in a dose-dependent induction of apoptosis. Addition of IGFs to the cells, alone or with C2, elicited no response in terms of proliferation or survival, respectively. When the cells were preincubated with ngIGFBP-3 before addition of C2 (2–5 μM), apoptosis was accentuated in a dose-dependent manner (at 100 ng/ml IGFBP-3, apoptosis increased from 11 to 88%).

In conclusion, we found that IGFBP-3 had no direct inhibitory effect on Hs578T cells but could accentuate apoptosis induced by the physiological trigger ceramide in an IGF-independent manner.

The insulin-like growth factors (IGF)1 -I and IGF-II are peptide mitogens for a number of different cell lines including human breast cancer cells (1, 2). The biological effects of both IGF-I and IGF-II appear to be mediated through the type 1 IGF receptor, which has a high affinity for both ligands. The availability of IGF is modulated by a family of IGF-binding proteins (IGFBPs), seven of which (IGFBP-1 to -7) have been cloned and sequenced to date (3, 4). Only a small fraction of unbound IGF-I and IGF-II remain in the circulation, the majority is bound to specific IGFBPs. IGFBP-3 is the major binding protein in human serum and serves as a carrier for the IGFs, prolonging their half-life in the circulation and maintaining an IGF reservoir (5). In addition IGFBP-3 is secreted by a number of cell types and can directly modulate IGF actions in tissues (6).

Both inhibiting and enhancing effects of IGFBP-3 on IGF-I action have been reported; inhibition appears to result from soluble IGFBP-3 sequestering IGF-I and preventing receptor interaction. One possible mechanism for potentiation of IGF-I action is by the cell surface binding of IGFBP-3 facilitating the binding of IGF-I to its receptor (7, 8).

There is also evidence to suggest an inhibitory role for IGFBP-3 that is independent of IGF-I. Purified mouse IGFBP-3 has been shown to bind to the chick embryo fibroblast cell surface and inhibit cell growth (9). Oh et al. (10, 11) have demonstrated the presence of specific IGFBP-3 binding sites on the surface of the breast cancer cells, Hs578T, and have shown that exogenous IGFBP-3 can inhibit monolayer growth in these cells by a mechanism that is IGF-independent. One of the aims of this study was to determine if the inhibitory action of IGFBP-3 in the Hs578T cells was as a result of apoptosis.

Cell growth is a balance between proliferation and programmed cell death (apoptosis). Apoptosis occurs in individual cells and is a strictly controlled, genetically regulated program operating under physiological conditions. It consists of a series of distinctive morphological changes: early nuclear compaction, cytoplasmic condensation followed by extensive nuclear fragmentation, after which the cell disintegrates into discrete membrane-bound apoptotic bodies that are engulfed by neighboring cells or phagocytic cells (12).

The p53 tumor suppressor gene is involved in the control of cell growth, and over-expression of this gene induces stimulation of cell death pathways (13, 14). Recent evidence indicates that p53 may regulate IGFBP-3 expression; over-expression of cellular p53 in EB1 colon carcinoma cells can induce the synthesis and secretion of a biologically active form of IGFBP-3 that can inhibit cell growth (15). The link between p53 and IGFBP-3 suggests a potential role for the latter in p53-mediated cellular growth control.

In addition to investigating the possibility that IGFBP-3 could induce apoptosis directly in Hs578T cells, there was also the possibility that it could modulate the signal pathway of a physiological trigger of programmed cell death. To investigate the latter, we established a cell model for inducible apoptosis. There are numerous agents available to induce apoptosis; however, not all act via physiological signaling pathways with which possible modulators, such as IGFBP-3, could interact. The sphingomyelin pathway in cells mediates the signaling for programmed cell death induced by radiation and several cytokines such as tumor necrosis factor α and interleukin-1β. These cytokines stimulate cell surface receptors activating a plasma membrane sphingomyelinase that hydrolyzes sphingomyelin to generate ceramide and phosphocholine. The ceramide then acts as a second messenger to activate the apoptotic process (16).
IGFBP-3 Enhances Apoptosis in a Non-IGF-dependent Manner

The precise downstream signaling events are presently unclear. Studies have shown that elevation of cellular ceramide levels by addition of synthetic ceramide analogues or bacterial sphingomyelinase mimicked tumor necrosis factor α action to induce apoptosis (17, 18).

In the present work, a synthetic ceramide analogue C2 was used to induce apoptosis in the human breast cancer cell line Ha578T. This enabled us to investigate the possibility that IGFBP-3-induced inhibition of cell growth was due to enhancement of a physiological signal pathway for programmed cell death.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human non-glycosylated IGFBP-3 (ngIGFBP-3) was a kind gift from Dr. C. Maack, Celtrix, CA. Recombinant human IGF-I and the IGF-I analogues DES 1–3 IGF-I and LR, IGF-1 were purchased from GroPep Adelaide, Australia. The ceramide analogue C2 was purchased from Sigma Corp. All other chemicals were purchased from Sigma Corp. Tissue culture plastics were obtained from Nunc, Life Technologies, Inc., Paisley, Scotland.

Cell Cultures—The human breast cancer cell line Ha578T was purchased from ECACC, Porton Down, Wiltsire, UK and were grown in a humidified 5% CO₂ atmosphere at 37 °C. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (5000 IU/ml), and streptomycin (5 mg/ml) growth media (GM). Experiments were performed on cells in serum-free HEPES, Dulbecco’s modified Eagle’s medium, and Ham’s nutrient mix F-12 (SFM) with sodium bicarbonate (0.12%), bovine serum albumin (0.2 mg/ml), transferrin (0.01 mg/ml), and supplemented as before.

The 3-(4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium Bromide) (MTT) Assay—The MTT assay is a colorimetric assay that crudely measures cell viability. MTT is a yellow-colored tetrazolium that is taken up and cleaved only by metabolically active cells, reducing it to a colored, water-insoluble formazan salt. Once the formazan has been solubilized, it can be easily and rapidly read in a conventional enzyme-linked immunosorbent assay plate reader at 595 nm. The absorbance directly correlates with cell number.

Cells were seeded at 5 × 10⁵ cells/ml (150 µl/well) in 96-well plates and grown for 24 h in GM. All plates were switched to SFM (100 µl/well) for 24 h prior to dosing. Plates were dosed as described under “Results” for the appropriate incubation times. Following this, MTT reagent (7.5 mg/ml) in phosphate-buffered saline was added (10 µl/well), and the cultures were incubated at 37 °C for 30 min. The reaction was stopped by the addition of acidified Triton buffer (0.1 M HCl, 10% (v/v) Triton X-100; 50 µl/well), and the tetrazolium crystals were dissolved by mixing in a Titertek plate shaker at room temperature for 20 min. The samples were measured on a Bio-Rad 450 plate reader at a test wavelength of 595 nm and a reference wavelength of 650 nm.

Results represent the mean ± S.E. of 5 wells from one experiment that is representative of experiments repeated at least three times. The results are optical density readings expressed as a percentage of controls.

Flow Cytometry and Cell Viability—Cells were seeded at 0.5 × 10⁶ cells/T25 flask and grown in GM for 24 h before switching to SFM for a further 24 h. The cells were dosed as described under “Results.” For analysis, both the floating cells in the supernatant and the phosphate-buffered saline wash were collected from each flask prior to trypsinization. Trypsinized cells were then added to the previous collected solutions. To assess cell viability, aliquots of cells were mixed with trypan blue (1:1) and loaded onto a hemocytometer, and the percentage of dead cells per sample was calculated. Each sample of cells was then fixed in 70% ethanol for a minimum of 24 h prior to analysis by flow cytometry. The fixed cells were pelleted (6000 rpm; 5 min) and washed three times with phosphate-buffered saline (6000 rpm; 5 min). The supernatant was removed, and the cells were resuspended in reaction buffer (0.05 mg/ml propidium iodide, 0.1% sodium citrate, 0.02 mg/ml RNase A, 0.3% Nonidet P-40, pH 8.3) and incubated at 4 °C for 30 min prior to measurement on a FACSCalibur flow cytometer (Beckton Dickinson) by an argon laser at 488 nm for excitation. The data were analyzed using the Cell Quest software package (Becton & Dickinson).

Morphological Assessment—To determine that C2-induced cell death gave rise to the classical morphological features associated with apoptosis, an aliquot of cells was taken from untreated and treated cells following the appropriate incubation times. The cells were cytospun and stained with the Wright stain in an automated stainer (19). Photos

![Fig. 1. Effects of exogenous IGF-I (A) and ngIGFBP-3 (B) on the metabolic activity of Ha578T cells after 24 h (●) and 48 h (○).](image)

Western Ligand Blotting and Immunoblotting (IB)—From the T25 flasks set up to determine flow cytometry and cell viability, the supernatants were collected and the cells spun out. The remaining solution was concentrated 10-fold using Millipore Ultrafree-MC filter units. The pattern of IGF-binding proteins secreted by the cells in response to various treatments and subsequent immunoblotting of the membranes for IGFBP-3 was determined using the methods described previously (20, 21). Briefly, the samples were separated by 12% SDS-polyacrylamide gel electrophoresis, and the proteins were then transferred onto a nylon membrane and probed with a mixture of 125I-labeled IGF-1 and 125I-labeled IGF-II. The same nylon membranes were then incubated with a specific polyclonal antibody for IGFBP-3 (SCH-2/6 at 1:15000) overnight at room temperature. Following the removal of excess unbound antibody, an anti-rabbit antibody conjugated to peroxidase (Sigma, at 1:10000) was added for 1 h. Binding of the peroxidase was visualized using enhanced chemiluminescence according to the manufacturer instructions (Amersham International).

Statistical Analysis—The data were analyzed using the Microsoft Excel Version 4.0a software package. Significant effects were determined using Student’s t-test. A statistically significant difference was considered to be present at p < 0.05.

RESULTS

We first investigated the response of the Ha578T cells to exogenous IGF-I and ngIGFBP-3 in the MTT assay. Fig. 1A shows that exogenously added IGF-I did not stimulate cell proliferation following 24 and 48 h incubation periods, even at...
grammed cell death, a number of different techniques were established that the cell death induced by C2 was due to programmed cell death as a pre-G1 peak on a DNA cell-cycle histogram. Fig. 4 illustrates the percentage of cells in the pre-G1 peak of each treated sample (0, 2, 4, 5, 7, and 10 μM C2). The results represent the mean ± S.E. for each C2 dose performed in triplicate in T25 flasks processed at the same time and are representative of experiments repeated at least three times. *, p < 0.005.

To establish that the decrease in metabolic activity seen with C2 was due to cell death, trypan blue uptake by dead cells was determined using a hemocytometer, and the percentage of dead cells in a given sample was calculated. Fig. 3 illustrates such an experiment in which cells were incubated with 0, 2, 4, 5, 7, or 10 μM concentrations of C2 for 24 h. It is evident that C2 causes a dose-dependent increase in the percentage of dead cells. The effect was significant from a dose of 4 μM C2 (p < 0.001). To establish that the cell death induced by C2 was due to programmed cell death, a number of different techniques were employed.

**Flow Cytometry**—This technique was used to quantitate the amount of apoptosis present in any given sample. Apoptotic cells have a lower DNA content than normal cells and appear as a pre-G1 peak on a DNA cell-cycle histogram. Fig. 4 illustrates the percentage of cells in the pre-G1 peak of samples treated with 0, 2, 4, 5, 7, or 10 μM C2 for 24 h. There was a dose-dependent increase in the percentage of apoptotic cells with increasing dose of C2. The 4 μM dose of C2 caused a significant (p < 0.05) increase in the percentage of cells in the pre-G1 phase of the cell cycle when compared with untreated cells.

**Morphological Features Observed**—Fig. 5A represents a normal untreated Hs578T cell. The progression of a cell through the different morphological stages of apoptosis, following treatment with 5 μM C2 for 24 h, is represented in Fig. 5, B-D. C2-induced apoptosis in Hs578T cells exhibits the classical morphological features of apoptosis.

**IGF-I Cannot Prevent C2-induced Cell Death**—To determine if IGF-I can prevent C2-induced cell death in Hs578T cells, the cells were incubated with C2 (1–30 μM) with or without 100 ng/ml IGF-I, 100 ng/ml DES 1–3 IGF-I, and 100 ng/ml LR3 IGF-I for 24 h and then assayed for MTT activity (Fig. 6). Both of the IGF-I analogues, DES 1–3 IGF-I and LR3 IGF-I, bind to the IGF-I receptor. However, DES 1–3 IGF-I only binds IGFBP-3 with a third of the affinity of IGF-I and LR3 IGF-I does not bind any of the binding proteins. From the figure, it is evident that IGF-I cannot prevent C2-induced cell death in Hs578T cells. In addition, this lack of an IGF-I effect is not due to interference from endogenous IGFBPs since the IGF analogues behaved in a similar manner to IGF-I.

**Preincubation with ngIGFBP-3 Accentuates the Apoptotic Response**—Even though ngIGFBP-3 alone had no marked inhibitory effect on Hs578T cell growth, we considered that it may be able to interact with the apoptotic pathway initiated by the ceramide analogue, C2, in these cells. This was examined at a low dose of C2 (2 μM), which elicited a minimal growth inhibitory effect in the MTT assay, and at a high dose (5 μM), which

![Fig. 2. The ceramide analogue, C2, induces a dose-dependent decrease in metabolic activity of Hs578T cells.](Image 99x569 to 257x729)

![Fig. 3. The ceramide analogue, C2, induces dose-dependent death in Hs578T cells.](Image 320x304 to 552x470)

![Fig. 4. The ceramide analogue, C2 induces a dose-dependent increase in apoptosis in Hs578T cells as measured by flow cytometry.](Image 320x553 to 552x729)
cells were coincubated with ngIGFBP-3 (1–100 ng/ml) and C2 (2 or 5 mM) for 24 h with or without a prior 24-h preincubation with 100 ng/ml ngIGFBP-3 and 5 mM C2, almost completely abolishing metabolic activity at both doses when 100 ng/ml ngIGFBP-3 was used.

**Accentuation of C2-induced Cell Death by ngIGFBP-3 Is Due to an Increase in Apoptosis**—To determine if ngIGFBP-3-accentuated C2 induced cell death by causing an increase in the amount of programmed cell death, cells treated with 0 or 4 mM C2, or coincubated with 4 mM C2 and 100 ng/ml ngIGFBP-3 with or without a 24-h preincubation with 100 ng/ml ngIGFBP-3 were analyzed for the pre-G1 apoptotic peak by flow cytometry (Fig. 8).

The 4 mM dose of C2 caused a significant ($p < 0.05$) increase in the percent of cells in the pre-G1 peak when compared with the control. The cells treated with the 24-h preincubation with 100 ng/ml ngIGFBP-3 prior to the coincubation with 4 mM C2 and 100 ng/ml ngIGFBP-3 have a highly significant increase in the percent of cells in the pre-G1 peak in comparison with both untreated ($p < 0.001$) and 4 mM C2-treated cells ($p < 0.001$).

**Immunoblotting for IGFBP-3**—Preliminary results indicate that after concentrating the conditioned media 10-fold, the Hs578T cells secrete just detectable traces of IGFBP-3 following addition of C2. With addition of exogenous ngIGFBP-3 to the Hs578T cells for 24 and 48 h, a number of IGFBP-3 fragments (13, 19, and 20 kDa) were seen in addition to the intact material in the conditioned media (data not shown). These results would suggest that the exogenously added ngIGFBP-3 is being processed by a protease produced by the cells.

**DISCUSSION**

To maintain tissue homeostasis, a balance between cell proliferation and programmed cell death is essential; in tumors this balance is obviously lost. The IGFs are the most prevalent growth factors in the body that, as well as being general mitogens, are also potent survival factors for many cells and can therefore impact on both aspects of this tissue balance.

Studies of transplantable tumors in vivo and of cell transformations in vitro have shown that activation of the IGF-I recep-
IGFBP-3 Enhances Apoptosis in a Non-IGF-dependent Manner

Fig. 7: Preincubation of Hs578T cells with ngIGFBP-3 accentuates the apoptotic effects of the ceramide analogue, C2. Cells were plated in 96-well plates, and all plates were switched to SFM 24 h prior to dosing. Fresh SFM was added to the plates that were to be dosed with ngIGFBP-3 alone or coincubated with ngIGFBP-3 and C2 (2 or 5 µM; panel B) for a further 24 h while the remaining plates were preincubated with ngIGFBP-3 (0.5–100 ng/ml) for a further 24 h (A). After this 24-h preincubation, all plates were decanted and redosed with either fresh ngIGFBP-3 alone (●) or coincubated with ngIGFBP-3 and 2 µM C2 (□) or 5 µM C2 (●) for a further 24 h. MTT activity was assayed as described under “Experimental Procedures.”

Fig. 8: ngIGFBP-3 accentuates the apoptotic effects of the ceramide analogue C2 in Hs578T cells by increasing the amount of apoptosis as measured by flow cytometry. Apoptotic cells appear as a pre-G1 peak, and the above graph illustrates the percentage of cells in the pre-G1, peak of each treated sample (untreated cells, cells treated with 100 ng/ml ngIGFBP-3, or 4 µM C2 alone or cells treated with and without a 24-h preincubation with 100 ng/ml ngIGFBP-3, followed by a coincubation with 4 µM C2 and 100 ng/ml ngIGFBP-3 for a further 24 h). The results represent the mean ± S.E. for each treatment performed in triplicate in T25 flasks processed at the same time and are representative of experiments repeated at least three times. *, p < 0.05; **, p < 0.001.

IGFBP-3 Enhances Apoptosis in a Non-IGF-dependent Manner

The p53 tumor suppressor gene is the most commonly mutated gene in human cancers (26). It is linked to growth regulatory processes including cell-cycle progression, DNA repair, and apoptosis (14). Recent studies have identified IGFBP-3 to be a p53-regulated target gene. Induction of IGFBP-3 expression by wild-type p53 leads to an active form of IGFBP-3 capable of inhibiting cell growth (15). This suggests that there may be a link between p53 and IGFBP-3 in regulating cellular growth, transformation, and survival.

In this study we have demonstrated that an analogue (C2) of ceramide, which is a physiological mediator of programmed cell death, induces apoptosis in Hs578T breast cancer cells as assessed by morphological criteria and flow cytometry. In these cells, exogenously added ngIGFBP-3 had only a small direct growth inhibitory effect. We have also shown that the Hs578T cells are nonresponsive to the IGFs in terms of both proliferation and cell survival. However, a preincubation with ngIGFBP-3 accentuated the apoptotic response of these cells to C2. These results indicate that IGFBP-3 can interact with the apoptotic pathway initiated by ceramide in an IGF-independent manner. The added IGFBP-3 was proteolytically modified by the cells, and it is not clear whether the enhanced apoptosis was effected by the intact IGFBP-3 or a fragment thereof. A fragment of IGFBP-3 has recently been reported to inhibit the growth of chick embryo fibroblasts (27). Thus IGFBP-3 may not only be a passive modulator of the IGF-induced mitogenic and survival signals but could also be an active regulator of a process that counterbalances these effects. IGF-I and IGFBP-3 are the most prevalent growth factor and the most prevalent binding protein, respectively; they are normally present in the body in association with each other in equimolar concentrations. This association may represent a balance between a survival signal and a signal for apoptosis; this balance may play an important role in tissue homestasis.

Acknowledgments—We are grateful to Dr A. E. Wakeling for helpful advice. We are also grateful to Dr C. Maack (Celtrix Pharmaceuticals, CA) for kindly providing the recombinant IGFBP-3.

The p53 tumor suppressor gene is the most commonly mutated gene in human cancers (26). It is linked to growth regulatory processes including cell-cycle progression, DNA repair, and apoptosis (14). Recent studies have identified IGFBP-3 to be a p53-regulated target gene. Induction of IGFBP-3 expression by wild-type p53 leads to an active form of IGFBP-3 capable of inhibiting cell growth (15). This suggests that there may be a link between p53 and IGFBP-3 in regulating cellular growth, transformation, and survival.

In this study we have demonstrated that an analogue (C2) of ceramide, which is a physiological mediator of programmed cell death, induces apoptosis in Hs578T breast cancer cells as assessed by morphological criteria and flow cytometry. In these cells, exogenously added ngIGFBP-3 had only a small direct growth inhibitory effect. We have also shown that the Hs578T cells are nonresponsive to the IGFs in terms of both proliferation and cell survival. However, a preincubation with ngIGFBP-3 accentuated the apoptotic response of these cells to C2. These results indicate that IGFBP-3 can interact with the apoptotic pathway initiated by ceramide in an IGF-independent manner. The added IGFBP-3 was proteolytically modified by the cells, and it is not clear whether the enhanced apoptosis was effected by the intact IGFBP-3 or a fragment thereof. A fragment of IGFBP-3 has recently been reported to inhibit the growth of chick embryo fibroblasts (27). Thus IGFBP-3 may not only be a passive modulator of the IGF-induced mitogenic and survival signals but could also be an active regulator of a process that counterbalances these effects. IGF-I and IGFBP-3 are the most prevalent growth factor and the most prevalent binding protein, respectively; they are normally present in the body in association with each other in equimolar concentrations. This association may represent a balance between a survival signal and a signal for apoptosis; this balance may play an important role in tissue homestasis.

Acknowledgments—We are grateful to Dr A. E. Wakeling for helpful advice. We are also grateful to Dr C. Maack (Celtrix Pharmaceuticals, CA) for kindly providing the recombinant IGFBP-3.
REFERENCES

1. De Leon, D., Bakker, B., Wilson, D. M., Lamson, G., and Rosenfeld, R. G. (1990) J. Clin. Endocrinol. Metab. 71, 530–532
2. Quinn, K. A., Treston, A. M., Unsworth, E. J., Miller, M.-J., Vos, M., Grimley, C., Battey, J., Mulshine, J. L., and Cuttitta, F. (1996) J. Biol. Chem. 271, 11477–11483
3. Shimazaki, S., Shimonaka, M., Zhang, H. P., and Ling, N. (1991) J. Biol. Chem. 266, 10646–10653
4. Oh, Y., Nagalla, S. R., Yamanaka, Y., Kim, H.-S., Wilson, E., and Rosenfeld, R. G. (1996) J. Biol. Chem. 271, 11477–11483
5. Neely, K., and Rosenfeld, R. (1992) Endocrinology 130, 985–993
6. Conover, C. A. (1991) Endocrinology 129, 3259–3268
7. Delbe, J., Villaudy, J., Blat, C., Desauty, G., Golde, A., and Harel, L. (1990) J. Cell. Physiol. 142, 359–364
8. Oh, Y., Muller, M. L., Lamson, G., and Rosenfeld, R. G. (1993) J. Biol. Chem. 268, 14964–14971
9. Oh, Y., Muller, M. L., Pham, H., and Rosenfeld, R. G. (1993) J. Biol. Chem. 268, 28045–28048
10. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
11. Cohen, J. J. (1993) Immunol. Today 14, 126–130
12. Levine, A. J. (1997) Cell 88, 323–331
13. Buckbinder, L., Talbott, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Seizinger, B. R., and Kley, N. (1995) Nature 377, 646–649
14. Obeid, L. M., Linardie, C. M., Karolak, L. A., and Hannun, Y. A. (1993) Science 259, 1769–1771
15. Obeid, L. M., and Hannun, Y. A. (1995) J. Cell. Biochem. 58, 191–198
16. Witty, J. P., Bridgham, J. T., and Johnson, A. L. (1996) Endocrinology 137, 5269–5277
17. Lillie, R. D. (1977) H. J. Conn’s Biological Stains, 9th Ed., pp. 605–606, Williams and Wilkins Co., Baltimore, MD
18. Coulson, V. J., Wass, J. A. H., Abdulla, A. F., Cotterill, A. M., and Holly, J. M. P. (1991) Growth Regulation 1, 119–124
19. Cwyfan-Hughes, S. C., Johnson, M. R., Heinrich, G., and Holly, J. M. P. (1995) J. Endocrinol. 147, 517–524
20. Resnicoff, M., Abraham, D., Yutananwiboonechai, W., Rotman, H. L., Kajstura, J., Rubins, Z., Zoltick, P., and Baserga, R. (1995) Cancer Res. 55, 2463–2469
21. Jones, J. I., and Clemmons, D. R. (1993) Endocrinol. Rev. 16, 3–34
22. Jones, J. I., and Clemmons, D. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10553–10557
23. Huynh, H., Yang, X., and Pollak, M. (1996) J. Biol. Chem. 271, 1016–1021
24. Holstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) Science 253, 49–53
25. Lalou, C., Lassarre, C., and Binoux, M. (1996) Endocrinology 137, 3206–3212