Autocrine Human Growth Hormone Inhibits Placental Transforming Growth Factor-β Gene Transcription to Prevent Apoptosis and Allow Cell Cycle Progression of Human Mammary Carcinoma Cells*

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Multiple cellular effects of human growth hormone (hGH) are mediated by an indirect mechanism requiring transcriptional activation of genes encoding protein effector molecules such as insulin-like growth factor-1. Such protein effector molecules then act directly to mediate the cellular functions of hGH. We report here that autocrine hGH production by mammary carcinoma cells specifically results in the transcriptional repression of the p53-regulated placental transforming growth factor-β (PTGF-β) gene. Transcriptional repression of the PTGF-β gene does not require the p53-binding sites in the PTGF-β promoter, and autocrine hGH also desensitized the response of the PTGF-β promoter to p53 overexpression. Transcriptional repression of the PTGF-β gene is accomplished by consequent decreases in its protein product, Smad-mediated transcription, and its cellular effects that include cell cycle arrest and apoptosis. PTGF-β specifically inhibited the autocrine hGH-stimulated expression of cyclin D1 required for autocrine hGH-stimulated mammary carcinoma cell cycle progression. Thus, one mechanism by which autocrine hGH promotes an increase in mammary carcinoma cell number is by transcriptional repression of protein effector molecules that promote cell cycle arrest and apoptosis. Such transcriptional repression of negative regulatory factors, such as PTGF-β, may also be requisite for direct stimulation of mammary carcinoma cell mitogenesis by hGH.

The human growth hormone (hGH)1 gene is expressed in epithelial cells of the normal human mammary gland.2 Increased epithelial expression of the hGH gene is associated with the acquisition of pathological proliferation, and the highest level of hGH gene expression is observed in metastatic mammary carcinoma cells.2 hGH receptor gene expression per mammary epithelial cell remains constant throughout the process of neoplastic progression (1), and therefore changes in the local concentration of ligand are likely to be pivotal to determine the effects of hGH on the behavior of the mammary epithelial cell. We have recently generated a model system to study the role of autocrine-produced hGH in mammary carcinoma by stable transfection of either the hGH gene or a translation-deficient hGH gene into mammary carcinoma (MCF-7) cells (2). The autocrine production of hGH by mammary carcinoma cells results in a hyperproliferative state with marked synergism observed between trophic agents such as IGF-1 (2). The increase in mammary carcinoma cell number as a consequence of autocrine production of hGH is a result of both increased mitogenesis and decreased apoptosis (3). Autocrine hGH production also results in enhancement of the rate of mammary carcinoma cell spreading on a collagen substrate (4), suggesting that it may affect cell motility and dissemination of the carcinoma. All of the studied effects of autocrine hGH on mammary carcinoma cell behavior are mediated via the hGH receptor (3). Thus, autocrine production of hGH by mammary carcinoma cells may direct mammary carcinoma cell behavior to impact on the final clinical prognosis. Systematic analysis of the relevant mechanistic features by which autocrine hGH exerts its cellular effects is therefore required.

One major mechanism by which GH affects cellular and somatic function is by regulating the level of specific mRNA species (5). Some of these GH-regulated genes code for trophic factors such as IGF-1 (6), which act in an intermediary role to execute the cellular effects of GH. Indeed, GH has been demonstrated to regulate the level of a number of trophic factors in specific tissues including hepatocyte growth factor in liver (7), epidermal growth factor in kidney (8), basic fibroblast growth factor in chondrocytes (9), interleukin-6 in osteoblasts (10), bone morphogenetic proteins 2 and 4 in fibroblasts (11), interleukin-1α and interleukin-1β in thymus (12), and preadipocyte factor-1 in adipocytes (13) and islet β-cells (14). It is therefore likely that many of the effects of autocrine hGH on mammary carcinoma cell function are also mediated by genetic regulation of specific trophic factors. Here we have used a cDNA microarray to identify autocrine hGH-regulated genes encoding polypeptide effector molecules that will act in an intermediary manner to mediate the effects of hGH on mammary carcinoma cell function.

We observed that autocrine hGH decreased transcription of...
the PTGF-β gene with consequent decreases in its protein product and accompanying cellular effects, which include cell cycle arrest and apoptosis. PTGF-β specifically inhibited the autocrine hGH-stimulated expression of cyclin D1 to prevent mammary carcinoma cell cycle progression. Thus, one mechanism by which autocrine hGH promotes mammary carcinoma cell survival is by transcriptional repression of protein effector molecules that promote cell cycle arrest and apoptosis. Such a mechanism is analogous and complementary to the ability of hGH to transcriptionally activate protein effector molecules, such as IGF-1, which stimulate processes resulting in increased cell expression.

**EXPERIMENTAL PROCEDURES**

**Materials—**Effectene™ transfection reagent and the One-step RT-PCR kit were obtained from Qiagen GmbH (Hilden, Germany). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT), and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium salt transfection reagent was from Roche Diagnostics. All other tissue culture's instructions and resuspended in diethyl pyrocarbonate-treated such as IGF-1, which stimulate processes resulting in in-autocrine hGH-stimulated expression of cyclin D1 to prevent expression in mammalian cells, the entire open reading frame of PTGF-β was disabled via a mutation to TTG generated by standard labeling for hybridization to the Atlas Human Cancer 1.2 Array—

**Analysis of Differential Gene Expression by Use of Atlas Human Cancer 1.2 Array—**MCF-7 cell line was obtained from the ATCC and stably transduced from Amersham Biosciences. TRI-REAGENT product and accompanying cellular effects, which include cell cycle arrest and apoptosis. PTGF-β/Luciferase construct (−1745CD1LUC) was a kind gift of Dr. Richard G. Lasky. Analysis of Differential Gene Expression by Use of Atlas Human Cancer 1.2 Array—

**RNA Gel Electrophoresis and Northern Blot Analysis—**The PTGF-β cDNAfragment was derived from the PTGF-β expression plasmid by digestion with the restriction enzyme EcoRI and purification of the fragment on an agarose gel. The DNA was then labeled with [α-32P]dCTP (3000 Ci/mm) using the Oligolabeling kit. In brief, 50 ng of DNA was denatured by heating for 2–3 min at 95°C and was directly transfected into MCF-7. MCF-7 cells were subcloned into the pGL-Basic-3 luciferase reporter plasmid as described previously (16). The Atlas Pure Total RNA Labeling system, Atlas Human Cancer 1.2 Array, and ExpressHyb solution were obtained from CLONTECH Laboratories (Palo Alto, CA). Hoechst 33258, denatured salmon testis DNA, and 5′-bromo-2′-deoxyuridine were purchased from Sigma.

The MCF-7 cell line was obtained from the ATCC and stably transduced with an expression plasmid containing the wild-type hGH gene (pMT-hGH) (18) under the control of the metallothionene 1a promoter (designated MCF-hGH) (2). For control purposes the ATG start site in the plasmid-hGH was disabled via a mutation to TTG generated by standard techniques (pMT-MUT) (18), and MCF-7 cells stably transfected with this plasmid were designated MCF-MUT (2). MCF-MUT cells therefore transcribe the hGH gene but do not translate the mRNA into protein. A polyclonal antibody against PTGF-β was generated as described previously (16). The Atlas Pure Total RNA Labeling system, Atlas Human Cancer 1.2 Array—

**Cell Culture—**MCF-7, MCF-hGH, and MCF-MUT cells (2) were cultured at 37°C in 5% CO2 in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm l-glutamine.

**Preparation of Total RNA—**Total RNA was isolated from MCF-MUT and MCF-hGH cells using TRI-REAGENT® according to the manufacturer's instructions and resuspended in diethyl pyrocarbonate-treated water. Quantification and purity of the RNA was assessed by Agarose gel electrophoresis. RNA samples with ratios greater than 1.6 were stored at −80°C for further analysis.

**Analysis of Differential Gene Expression by Use of Atlas Human Cancer 1.2 Array—**Poly(A)+ RNA was reverse-transcribed in the presence of [α-32P]dATP for generation of radiolabeled cDNA. Probes were purified and hybridized to the Atlas Human Cancer 1.2 Array according to the manufacturer's instructions (overnight at 68°C). After a series of high stringency washes (three 20-min washes in 2× saline/sodium citrate hGH (MSC), 50% SDS followed by two 20-min washes in 1× SSC, 0.5% SDS) at 68°C, the membranes were exposed to x-ray film and subjected to autoradiography. The relative levels of gene expression were quantified by densitometric scanning by use of the GS-700 imaging densitometer from Bio-Rad according to the manufacturer's instructions. Genes were considered differentially expressed when they exhibited a 2-fold or greater increase or decrease in the presence of autocrine hGH (MCF-MUT cells) compared with the absence of autocrine hGH (MCF-hGH cells) in three independently performed experiments. The relative expression of housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, β-actin, and ribosomal protein S9) served to normalize gene expression levels and did not differ by more than 10% between MCF-MUT and MCF-hGH cells.

**Probe Labeling for Northern Blot Analysis—**The PTGF-β cDNA fragment was derived from the PTGF-β expression plasmid by digestion with the restriction enzyme EcoRI and purification of the fragment on an agarose gel. The DNA was then labeled with [α-32P]dCTP (3000 Ci/mm) using the Oligolabeling kit. In brief, 50 ng of DNA was denatured by heating for 2–3 min at 95°C and was directly transfected into MCF-7. MCF-7 cells were subcloned into the pGL-Basic-3 luciferase reporter plasmid as described previously (16). The Atlas Pure Total RNA Labeling system, Atlas Human Cancer 1.2 Array, and ExpressHyb solution were obtained from CLONTECH Laboratories (Palo Alto, CA). Hoechst 33258, denatured salmon testis DNA, and 5′-bromo-2′-deoxyuridine were purchased from Sigma.

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**Preparation of Total RNA—**Total RNA was isolated from MCF-MUT and MCF-hGH cells using TRI-REAGENT® according to the manufacturer's instructions and resuspended in diethyl pyrocarbonate-treated water. Quantification and purity of the RNA was assessed by Agarose gel electrophoresis. RNA quality was assessed by agarose gel electrophore-

**Gene Expression**

**Analysis of Differential Gene Expression by Use of Atlas Human Cancer 1.2 Array—**Poly(A)+ RNA was isolated from total RNA using the Atlas Pure Total RNA labeling system. Three independently derived total RNA samples from the respective cell lines were pooled before labeling for hybridization to the Atlas Human Cancer 1.2 Array.
Luciferase Reporter Assay for PTGF-β and Cyclin D1 Promoter Constructs—MCF cells were cultured to 80% confluence in 6-well plates. Transient transfection was performed in serum-free RPMI with Effectene according to the manufacturer's instructions. 0.2 μg of PTGF-β expression plasmid or the control vector were transfected per well in serum-free RPMI medium for 12 h before the medium was changed to fresh serum-free RPMI. After 48 h cells were lysed at 4 °C in 150 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 0.2% phenylmethylsulfonyl fluoride) for 30 min with regular vortices. Cell lysates were then centrifuged at 14,000 × g for 15 min; the resulting supernatants were collected and subjected to 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a standard semidry method, using bovine serum albumin as a standard. 5× SDS sample buffer (50 mM Tris-HCl, pH 6.6, 5% SDS, 5% β-mercaptoethanol, and bromophen blue) was added to 20 μg of total protein, boiled for 5 min, and centrifuged at 14,000 × g for 5 min. The supernatants were collected and subjected to 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membrane using a standard semidyed electrophoresis apparatus in Laemmli buffer containing 10% methanol.

For the analysis of the levels of secreted PTGF-β protein, MCF-MUT and MCF-hGH cells were grown in serum-free medium. Medium was collected, and 400 μl were precipitated with acetone at −20 °C for 2 h, centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant discarded. The pellet was washed once with 70% methanol and centrifuged at 16,000 × g for 15 min at 4 °C. The pellet was boiled in 5× SDS sample buffer for 5 min and centrifuged at 14,000 × g for 5 min. The supernatants were collected and subjected to 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a standard semidyed electrophoresis apparatus in Laemmli buffer containing 10% methanol.

Nitrocellulose membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline with 0.1% Tween 20 (PBST) for 2 h at 22 °C. The membranes were then treated for 1 h at 22 °C with the primary antibody in PBST containing 1% non-fat dry milk (anti-PTGF-β 1:500, anti-β-catenin 1:1000, anti-β-actin 1:1000, PARP-1 1:1000, anti-cyclin D1 1:1000, anti-p27Kip1 1:2500, and anti-p21Waf/Cip1 1:500). After 3 washes with PBST, membranes were incubated in either goat anti-mouse or goat anti-rabbit IgG (1:4000) horseradish peroxidase-conjugated second antibodies for 1 h at 22 °C. The membranes were washed with PBST and incubated with 5% non-fat dry milk in PBST for 1 h at 22 °C with the primary antibody in PBST containing 1% non-fat dry milk (anti-PTGF-β 1:500, anti-β-catenin 1:1000, anti-β-actin 1:1000, PARP-1 1:1000, anti-cyclin D1 1:1000, anti-p27Kip1 1:2500, and anti-p21Waf/Cip1 1:500). After 3 washes with PBST, membranes were incubated in either goat anti-mouse or goat anti-rabbit IgG (1:4000) horseradish peroxidase-conjugated secondary antibodies for 1 h at 22 °C. Membranes were further washed in PBST before immunolabeling was detected by ECL according to the manufacturer's instructions.

Western Blot Analysis—MCF-MUT and MCF-hGH cells were grown to 80% confluence in 6-well plates. Transient transfection was performed in serum-free RPMI with Effectene according to the manufacturer's instructions. 0.2 μg of PTGF-β expression plasmid or the control vector were transfected per well in serum-free RPMI medium for 12 h before the medium was changed to fresh serum-free RPMI. After 48 h cells were lysed at 4 °C in 150 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 0.2% phenylmethylsulfonyl fluoride) for 30 min with regular vortices. Cell lysates were then centrifuged at 14,000 × g for 15 min; the resulting supernatants were collected and subjected to 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a standard semidyed electrophoresis apparatus in Laemmli buffer containing 10% methanol.

Measurement of apoptosis—Apoptotic cell death was measured by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst 33258 (21). To prepare PTGF-β conditioned medium for determination of apoptosis, subconfluent MCF-7 cells were transfected with the PTGF-β expression plasmid or for control with the empty vector in serum-free medium for 12 h and serum-starved for 24 h. The media were collected from three transfection experiments and spun down for 5 min at 600 × g, and the supernatant was collected.

MCF-MUT and MCF-hGH cells were trypsinized with 0.5% trypsin and washed twice with serum-free RPMI medium. The cells were then seeded to glass cover slips in 6-well plates and incubated in serum-free RPMI medium and transfected with either a control vector or an expression vector containing PTGF-β cDNA. After a culture period of 24 h in serum-free RPMI medium, the cells were fixed for 20 min in 4% paraformaldehyde in PBS, pH 7.4, at room temperature. Alternatively, cells were cultured in the presence of conditioned media. The cells were then rinsed twice in PBS and stained with the karyophilic dye Hoechst 33258 (20 μg/ml) for 5 min at room temperature. Following washing with PBS, nuclear morphology was examined under a UV-visible fluorescence microscope (Zeiss Axiosplan). Apoptotic cells were distinguished from viable cells by their nuclear morphology characterized by nuclear condensation and fragmentation as well as the higher intensity of the blue fluorescence of the nuclei. For statistical analysis, three times 300 cells were counted in eight random microscopic fields at ×400 magnification.

Statistics—All experiments were repeated at least three to five times. All numerical data are expressed as mean ± S.E., and the data were analyzed using Instat 3.0 from GraphPad Software Inc.

RESULTS

Identification of PTGF-β as a Gene Negatively Regulated by the Autocrine Production of hGH in Human Mammary Carcinoma Cells—To identify genes regulated by autocrine production of hGH in mammary carcinoma cells, we screened a high density cDNA array with labeled cDNA derived from either MCF-7 cells stably transfected with the hGH gene but with the start codon mutated to TTG (MCF-MUT) (A) or in MCF-7 cells stably transfected with the hGH gene (MCF-hGH) (B) cultured in serum-free medium. 32P-Labeled cDNA probes generated from poly(A)+ RNA isolated from MCF-MUT and MCF-hGH cells were hybridized to a cDNA microarray containing 1176 known human genes. The position of PTGF-β (MIC-1) cDNA is indicated by the arrow. The relative expression level of specific cDNAs was determined by comparison with the expression of a number of housekeeping genes, and one (tubulin α1 subunit) is indicated on the membrane.

Use of Cyclin D1 Antisense Oligonucleotide to Deplete Cellular Cyclin D1—The 20-mer cyclin D1 sense and antisense oligonucleotides utilized were as published previously (20) and were completely modified with phosphorothioate: for cyclin D1 sense, 5′-CCACGGCATGGACAGACGAGC-3′, and antisense, 5′-GTCGTTGGTCCATGCTGG-3′. Cells were transfected with 800 nM oligonucleotide for 8 h in N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium salt (10 μM/ml medium). Cells were either lysed and processed as described above for Western blot analysis or processed for determination of nuclear BrdUrd incorporation (as described above).

Measurement of Apoptosis—Apoptotic cell death was measured by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst 33258 (21). To prepare PTGF-β conditioned medium for determination of apoptosis, subconfluent MCF-7 cells were transfected with the PTGF-β expression plasmid or for control with the empty vector in serum-free medium for 12 h and serum-starved for 24 h. The media were collected from three transfection experiments and spun down for 5 min at 600 × g, and the supernatant was collected.

MCF-MUT and MCF-hGH cells were trypsinized with 0.5% trypsin and washed twice with serum-free RPMI medium. The cells were then seeded to glass cover slips in 6-well plates and incubated in serum-free RPMI medium and transfected with either a control vector or an expression vector containing PTGF-β cDNA. After a culture period of 24 h in serum-free RPMI medium, the cells were fixed for 20 min in 4% paraformaldehyde in PBS, pH 7.4, at room temperature. Alternatively, cells were cultured in the presence of conditioned media. The cells were then rinsed twice in PBS and stained with the karyophilic dye Hoechst 33258 (20 μg/ml) for 5 min at room temperature. Following washing with PBS, nuclear morphology was examined under a UV-visible fluorescence microscope (Zeiss Axiosplan). Apoptotic cells were distinguished from viable cells by their nuclear morphology characterized by nuclear condensation and fragmentation as well as the higher intensity of the blue fluorescence of the nuclei. For statistical analysis, three times 300 cells were counted in eight random microscopic fields at ×400 magnification.

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RESULTS

Identification of PTGF-β as a Gene Negatively Regulated by the Autocrine Production of hGH in Human Mammary Carcinoma Cells—To identify genes regulated by autocrine production of hGH in mammary carcinoma cells, we screened a high density cDNA array with labeled cDNA derived from either MCF-7 cells stably transfected with the hGH gene but with the start codon mutated to TTG (MCF-MUT) (A) or in MCF-7 cells stably transfected with the hGH gene (MCF-hGH). We have detailed previously (2, 4) the extensive characterization of these two cell lines. We used the CLONTECH Atlas Human Cancer 1.2 array and were particularly examining for factors, which inhibited proliferation or promoted cell death. One gene that we observed to be consistently decreased in MCF-hGH cells in comparison to MCF-MUT cells was macrophage inhib-
mRNA in MCF-MUT and MCF-hGH cells by semi-quantitative RT-PCR. The validation that the conditions of RT-PCR used here yielded semi-quantitative estimates of mRNA is described under “Experimental Procedures” and has been utilized previously (24).

One amplified fragment of the predicted size (368 bp) appropriate for PTGF-β mRNA was detected in MCF-MUT and MCF-hGH cells (Fig. 2A). Autocrine production of hGH by MCF-hGH cells resulted in a decreased level of PTGF-β mRNA in comparison to MCF-MUT cells. The level of β-actin mRNA did not differ between the two cell lines and was used as a control for RNA quality (Fig. 2A).

To verify further that autocrine hGH production in mammary carcinoma cells resulted in a decreased level of PTGF-β mRNA, we resorted to Northern blot analysis. PTGF-β mRNA of the appropriate size (1.2 kb) was detected in both MCF-MUT and MCF-hGH cells. Autocrine production of hGH by MCF-hGH cells resulted in a decreased level of PTGF-β mRNA in comparison to MCF-MUT cells when cultured in serum-free medium. Stimulation of either MCF-MUT or MCF-hGH cells with 50 nM exogenous hGH did not significantly alter the level of PTGF-β mRNA. Interestingly, growth of either MCF-MUT or MCF-hGH cells with 10% FBS dramatically increased the level of PTGF-β mRNA although the level of PTGF-β mRNA tended to be lower in the MCF-hGH cell line. The expression of β-actin mRNA remained relatively constant between MCF-MUT and MCF-hGH cells under the different experimental conditions (Fig. 2A).

Effect of Autocrine hGH, Exogenous hGH, and FBS on Luciferase Activity from a Reporter Plasmid Containing the Promoter Region of the PTGF-β Gene—To determine whether the autocrine hGH stimulated decrease in PTGF-β mRNA was due to down-regulation of PTGF-β gene transcription, we utilized a luciferase reporter plasmid containing the promoter region (923 bp) of the PTGF-β gene (16). Autocrine hGH production by MCF-hGH cells decreased luciferase expression 3–4-fold to that observed in MCF-MUT cells (Fig. 3A). Exogenous hGH (50 nM) did not affect PTGF-β gene transcription by MCF-MUT cells nor did it further repress transcription of the PTGF-β gene in MCF-hGH cells. 10% FBS resulted in a slight enhancement of PTGF-β gene transcription in MCF-MUT cells, and this enhancement of PTGF-β gene transcription by 10% FBS was similarly abrogated by autocrine production of hGH in MCF-hGH cells.

To exclude definitively the possibility that the differences in PTGF-β gene transcription observed between MCF-MUT and MCF-hGH cell lines were due to clonal selection artifact, we examined the response of PTGF-β gene transcription in parental MCF-7 cells to transient transfection of the hGH gene. For control purposes the ATG start site in pMT-hGH was disabled via a mutation to TTG, and this construct served as the control vector (as for MCF-MUT cells; pMT-MUT). As observed in Fig. 3B transient transfection of the hGH gene in MCF-7 cells similarly reduced PTGF-β gene transcription in comparison to MCF-7 cells transiently transfected with the control vector. Exogenous hGH (either 50 or 100 nM) did not affect PTGF-β gene transcription in MCF-7 cells as was observed for exogenous hGH applied to MCF-MUT cells. Thus PTGF-β is one gene selectively regulated by the autocrine production of hGH and is not co-regulated by exogenous hGH.

Interaction of Autocrine Production of hGH by Mammary Carcinoma Cells with hIGF-1 and 17β-Estradiol (E2) on the Level of PTGF-β Gene Transcription—We have demonstrated previously (2) that autocrine hGH production by mammary carcinoma cells synergizes with exogenous administration of hIGF-1 but not E2 to increase cell number. We therefore first examined the effect of 10 nM exogenously added hIGF-1 on
PTGF-β gene transcription in MCF-MUT and MCF-hGH cells. Treatment of MCF-MUT cells with hIGF-1 increased luciferase activity from the reporter construct containing the promoter region of the PTGF-β gene, indicating that hIGF-1 increased transcription of the PTGF-β gene (Fig. 4A). However, hIGF-1 did not significantly release the inhibition of PTGF-β gene transcription due to autocrine production of hGH in MCF-hGH cells. Thus, autocrine hGH exerted the dominant effect on PTGF-β gene transcription. Treatment of both MCF-MUT and MCF-hGH cells with E2 did not consistently alter transcription of the PTGF-β gene although there was a slight tendency to increase reporter activity in some experiments (Fig. 4B).

Autocrine hGH Inhibition of PTGF-β Gene Transcription Is p53-independent—PTGF-β has been identified as a p53 target gene (16). We therefore examined whether autocrine hGH inhibition of PTGF-β gene transcription involved a p53-dependent component. p53 has been demonstrated previously to bind to two p53-binding sites present in the promoter region of the PTGF-β gene utilized here (16). These are located at −898 to −879 and −43 to −24 upstream from the putative translation initiation site (16). Deletion of both p53-binding sites in the PTGF-β gene promoter dramatically reduced transcription of the PTGF-β gene (observe the relative comparative values between luciferase activity generated from the wild-type promoter and the p53-binding site deleted promoter in Fig. 5A and Fig. 5A). Despite the dramatic reduction in reporter activity upon deletion of the p53-binding sites in the PTGF-β gene promoter, the autocrine production of hGH by MCF-hGH cells maintained the equivalent inhibition of PTGF-β gene transcription (Fig. 5A). Autocrine production of hGH by MCF-hGH cells also abrogated the enhanced response of the intact PTGF-β promoter to overexpression of wild-type p53 (Fig. 5A). Furthermore, the inhibitory effect of autocrine production of hGH by MCF-hGH cells on the PTGF-β promoter was maintained when several PTGF-β gene promoter inactive (p53-mu143A and p53-mu273H) or active (p53-mu281G) mutants of p53 (16) were utilized (Fig. 5B). Thus autocrine hGH inhibition of PTGF-β gene transcription in mammary carcinoma cells does not involve p53.

Effect of Autocrine hGH on the Level of PTGF-β Protein in Mammary Carcinoma Cells—PTGF-β is a secreted protein and is secreted to the media in both a premature 40-kDa form and

**Fig. 3.** Effect of autocrine hGH and exogenous hGH and FBS on luciferase activity from a reporter plasmid containing the promoter region (923 bp) of the PTGF-β gene. A, MCF-MUT and MCF-hGH cells in serum-free media or in serum-free media supplemented with 50 nM hGH or 10% FBS or both were transiently transfected with the respective plasmids (0.2 µg of PTGF-β-w/p53BS and 0.2 µg of pCMVβ), and luciferase assays were performed as described under “Experimental Procedures.” B, MCF-7 cells in serum-free media or in serum-free media supplemented with 50 nM hGH were transiently transfected with the respective plasmids (0.2 µg of PTGF-β-w/p53BS and 0.2 µg of pCMVβ) or either 0.2 µg of pMT-MUT or pMT-hGH and 0.2 µg of pCMVβ, and luciferase assays were performed as described under “Experimental Procedures.” Where indicated exogenous hGH was used at 50 nM final concentration. Results are presented as the relative luciferase activity normalized to constitutive β-galactosidase expression. Values are depicted as means ± S.E. from three independent experiments. *, p value of <0.05; **, p value of <0.01; *** p value of <0.001 based on ANOVA, followed by Bonferroni multiple comparison test. A, ANOVA, followed by Bonferroni multiple comparison test; B, unpaired t test.

**Fig. 4.** Interaction of 17β-estradiol and insulin-like growth factor-1 with autocrine hGH on transcription of the PTGF-β gene. MCF-MUT and MCF-hGH cells were grown to confluence in serum-free media or in serum-free media supplemented with either human insulin-like growth factor-1 (A) or 17β-estradiol (B) and were transiently transfected with the respective plasmids (0.2 µg of PTGF-β-w/p53BS and 0.2 µg of pCMVβ). Luciferase assays were performed as described under “Experimental Procedures.” Results are presented as the relative luciferase activity normalized to constitutive β-galactosidase expression and are given as means ± S.E. of triplicate determinations. *, p value of <0.05; **, p value of <0.01; *** p value of <0.001 based on ANOVA, followed by a Bonferroni multiple comparison test.
a mature 15-kDa form (22). To determine whether the autocrine hGH inhibition of PTGF-β gene transcription also resulted in decreased PTGF-β protein, we examined the level of PTGF-β in media collected from MCF-MUT and MCF-hGH cells by Western blot analysis. Both the premature PTGF-β at 40 kDa and the mature PTGF-β at 15 kDa could be detected by Western blot analysis in media from MCF-MUT and MCF-hGH cells (Fig. 6A). However, MCF-hGH cells secreted significantly less of both the premature and mature forms of PTGF-β to the media. Thus autocrine hGH production by mammary carcinoma cells also significantly decreases PTGF-β production.

**Effect of Autocrine hGH on Smad-mediated Transcriptional Activation in Mammary Carcinoma Cells**—It has been demonstrated previously (16, 25) that PTGF-β functions through the TGF-β receptor to activate Smad-mediated transcription. Because autocrine production of hGH by MCF-hGH cells decreased the level of PTGF-β, it could be expected that MCF-hGH cells would also exhibit a decrease in Smad-mediated transcription in comparison to MCF-MUT cells. We therefore used an artificial luciferase reporter construct containing four tandem repeats of the Smad3/4-binding consensus sequence (SBE, GTCTAGAC) (26) to examine the level of Smad-mediated transcription in comparison to MCF-MUT cells. We therefore used an artificial luciferase reporter construct containing four tandem repeats of the Smad3/4-binding consensus sequence (SBE, GTCTAGAC) (26) to examine the level of Smad-mediated transcription in MCF-MUT and MCF-hGH cells. Smad-mediated transcriptional activity was present in MCF-MUT cells designated as 100%. Cells were cultured to confluency and transiently transfected with the 4× SBE reporter plasmid, and luciferase assays were performed as described under “Experimental Procedures.” Results are presented as the relative luciferase activity normalized to constitutive α-galactosidase expression and are given as means ± S.E. of triplicate determinations. *, p value of <0.05; **, p value of <0.01; and ***, p value of <0.001 based on ANOVA, followed by a Bonferroni multiple comparison test.

**Effect of Forced Expression PTGF-β on MCF-MUT and MCF-hGH Cell Proliferation**—We have demonstrated previously (2) that autocrine production of hGH by mammary carcinoma cells increased cell proliferation. The D family of cyclins is pivotal to initiate progression through the G1 phase of the cell cycle (27). Cyclin D1 is the predominant member of this family expressed in mammary gland (28) and is primarily regulated at the tran...
nucleus, unpaired

**Fig. 7.** Effect of forced expression of PTGF-β on autocrine hGH-stimulated cell cycle progression. A, effect of autocrine hGH on cyclin 1 promoter activity. MCF-MUT and MCF-hGH cells in serum-free media were transiently transfected with a cyclin D1 promoter luciferase construct (−1745CDILUC) and either a control vector or an expression vector containing PTGF-β cDNA. Luciferase assays were performed as described under “Experimental Procedures.” B, Western blot analysis to detect the effect of forced expression of PTGF-β on p21Waf1/Cip1, p27Kip1, and cyclin D1 expression in MCF-MUT and MCF-hGH cells. MCF-MUT and MCF-hGH cells were grown to confluence and transfected in serum-free media with either control vector or an expression vector containing PTGF-β cDNA. Cell extracts were prepared and subjected to SDS-PAGE, and Western blot analysis was performed as described under “Experimental Procedures.” After visualization of p21Waf1/Cip1 and p27Kip1 expression, the membrane was subsequently stripped and reblotted for β-actin to demonstrate equivalent loading. C, the effect of forced expression of PTGF-β on BrdUrd incorporation in MCF-MUT and MCF-hGH cells in serum-free media. MCF-MUT and MCF-hGH cells were transfected in serum-free media with either control vector or an expression vector containing PTGF-β cDNA and processed for BrdUrd incorporation as described under “Experimental Procedures.” D, the effect of PTGF-β conditioned media on BrdUrd incorporation in MCF-MUT and MCF-hGH cells in serum-free media. PTGF-β cDNA was transfected in MCF-7 cells, and conditioned media were collected and diluted until PTGF-β was present at the same concentration as from MCF-MUT cells. Control or PTGF-β-conditioned media was applied to MCF-MUT and MCF-hGH cells for 24 h and processed for BrdUrd incorporation as described under “Experimental Procedures.” A, the results are given as means ± S.E. of triplicate determinations, and asterisks indicate a p value of <0.01 based on ANOVA, followed by a Bonferroni multiple comparison. C and D, the results represent means ± S.E. of triplicate determinations of the percentage of cells incorporating BrdUrd in the nucleus, unpaired t test, p < 0.001.

The expression level by changes in promoter activity (53). We therefore first examined the effect of autocrine production of hGH by mammary carcinoma cells on the activity of the cyclin D1 promoter (17). Autocrine production of hGH in MCF-hGH cells resulted in a tripling of the promoter activity (17). We subsequently examined the level of cyclin D1 protein in MCF-hGH compared with MCF-MUT cells. Forced expression of PTGF-β in MCF-MUT cells resulted in an increase in p21 Waf1/Cip1 protein levels in comparison to MCF-MUT cells (Fig. 7B). Equal loading of the cell extracts was verified by reprobing the stripped membrane for β-actin (Fig. 7B). Forced expression of PTGF-β in MCF-MUT cells resulted in an increase in p21Waf1/Cip1 protein as could be expected (52), but no effect of forced expression of PTGF-β on the level of p27Kip1 protein in MCF-hGH cells was observed (Fig. 7B). We next examined the protein level of the cdk inhibitor p27Kip1. Decreased expression of p27Kip1 is associated with G1/S phase transition (29). The level of p27Kip1 was decreased in MCF-hGH cells compared with MCF-MUT cells concordant with increased cell cycle progression of MCF-hGH cells in comparison to MCF-MUT cells (see below). Forced expression of PTGF-β did not significantly alter the expression level of p27Kip1 in MCF-hGH cells (Fig. 7A). To determine the effects of forced expression of PTGF-β on pro-
transfection of cyclin D1 antisense oligonucleotides in MCF-hGH also reduced the percentage of cells with nuclear BrdUrd incorporation to that observed in MCF-MUT cells (Fig. 8B). Thus, an autocrine hGH-stimulated increase in cyclin D1 is required for autocrine hGH-stimulated cell cycle progression. Therefore, PTGF-β inhibition of autocrine hGH-stimulated cyclin D1 expression in mammary carcinoma cells is sufficient to prevent autocrine hGH-stimulated cell cycle progression.

**Effect of PTGF-β on MCF-MUT and MCF-hGH Cell Survival**—We have demonstrated previously (3, 24) that autocrine production of hGH in MCF-hGH cells affords dramatic protection from apoptotic cell death in comparison to MCF-MUT cells. In contrast, exogenous hGH only marginally reduces the level of apoptosis of MCF-MUT cells in serum-free media (3). It can therefore be suggested that down-regulation of PTGF-β by autocrine hGH in mammary carcinoma cells is responsible for decreased apoptotic cell death in MCF-hGH cells compared with MCF-MUT cells. We first examined the effect of transient overexpression of PTGF-β on the formation of the 60-kDa β-catenin cleavage product which is associated with caspase activation and subsequent apoptosis (31, 32). A 60-kDa cleavage product for β-catenin could be detected in both MCF-MUT and MCF-hGH cells (Fig. 9A). Forced expression of PTGF-β in both MCF-MUT and MCF-hGH resulted in a dramatic increase in the level of the 60-kDa β-catenin cleavage product. We also examined the effect of forced expression of PTGF-β on the formation of a caspase 3-dependent 85-kDa cleavage product from PARP (33). Western blot analysis for PARP expression and cleavage demonstrated a dramatically increased expression of PARP in MCF-hGH compared with MCF-MUT cells (Fig. 9A). Forced expression of PTGF-β in both MCF-MUT and MCF-hGH cells did not result in a detectable cleavage product. This is concordant with the observation that PARP cleavage occurs as a result of caspase 3 activation (32). MCF-7 cells are caspase 3-deficient (34) and are subject to caspase 3-independent apoptosis (35). We were also not able to detect caspase 3 activity nor PTGF-β-stimulated caspase 3 activity in MCF-MUT and MCF-hGH cells with a specific caspase 3 activity assay (data not shown). Concordant with the increased level of the 60-kDa β-catenin cleavage product upon forced expression of PTGF-β, forced expression of PTGF-β also dramatically increased apoptotic cell death in both MCF-MUT and MCF-hGH cells, with the apoptotic rate of MCF-hGH cells transfected with PTGF-β cDNA similar to that observed in MCF-MUT cells in serum-free media. PTGF-β conditioned media containing PTGF-β at the level of MCF-MUT cells derived as described above also dramatically increased the percentage of apoptotic cells in both MCF-MUT and MCF-hGH cell lines, with the level of apoptosis in MCF-hGH cells once again approximating that in MCF-MUT cells (Fig. 9C). Thus, PTGF-β promotes apoptotic cell death in mammary carcinoma cells, and down-regulation of PTGF-β gene transcription by hGH is one mechanism by which autocrine hGH prevents apoptotic cell death.

**DISCUSSION**

We have demonstrated here that autocrine production of hGH by mammary carcinoma cells results in a specific decrease of PTGF-β gene transcription. Thus, autocrine hGH function in the mammary epithelial cell, such as mitogenesis and cell survival, may be partly mediated by, or first require, repression of the inhibitory effects of PTGF-β. Furthermore, because PTGF-β is a secreted protein, it will also act in a paracrine fashion to affect the function of neighboring cells. In carcinoma of the mammary gland, such paracrine interactions usually occur between carcinoma cells of epithelial origin and neighboring stromal fibroblasts and are pivotal to the development of carcinoma (36). It is noteworthy to mention that the hGH
For example, fibroadenoma of the mammary gland, where we observe increased expression of the hGH gene in epithelial cells, exhibits an intense stromal reaction. It is possible that loss of paracrine PTGF-β secretion by the mammary epithelial cell in response to autocrine hGH production may contribute to changes in stromal architecture. TGF-β secreted by mammary carcinoma cells has been demonstrated to affect stromal architecture and tumor progression (36). Use of in vivo models will allow us to define the effect of autocrine production of hGH by mammary epithelial or mammary carcinoma cells on the surrounding stroma and delineate the relative contribution to neoplastic progression.

PTGF-β is a recently identified secretory protein that shares ~25% sequence identity with TGF-β family members (23) and possesses several characteristics of the TGF-β superfamily, including a signal peptide, a consensus RXR(A/S) cleavage signal for processing to the mature form, and seven conserved cysteine residues in the carboxyl terminus (mature form) (22, 23, 37–39). PTGF-β has also been demonstrated to utilize either the type I TGF-β receptor or type II TGF-β receptor to mediate cell cycle arrest (16). Activation of type I TGF-β receptor or type II TGF-β receptor results in a complex series of downstream signaling events resulting in phosphorylation of Smad proteins that translocate to the nucleus, associate with transcriptional co-activators, and transactivate TGF-β-regulated genes (40). We also observed here that autocrine production of hGH by mammary carcinoma cells results in decreased Smad-mediated gene transcription in accord with the decreased production of PTGF-β. Other mechanisms may also exist for the observed autocrine hGH-stimulated decrease in Smad-mediated transcription. By use of cDNA microarray technology we have reported recently (24) that the ski oncogene is ~4-fold up-regulated in response to autocrine production of hGH. It has been demonstrated recently that ski associates with both Smad2 and Smad3 resulting in repression of TGF-β-responsive promoters via the Smad-binding element (SBE) used here (41). The TGF-β pathway usually functions to suppress cellular proliferation and cellular transformation. It has been proposed that the repression of TGF-β-inducible genes (which function as negative regulators of cell cycle function in mammary epithelial cells) may be pivotal to the cellular transforming ability of ski (41). Thus, autocrine hGH production by mammary carcinoma cells and other induce negative regulators and suppress positive regulators of the TGF-β pathway. That autocrine production of hGH by mammary carcinoma cells results in such coordinated repression of the TGF-β axis is suggestive that many functions of autocrine hGH in mammary epithelial cells may be achieved by simple antagonism of this pathway.

PTGF-β was identified as a p53-regulated gene (16, 25). We have demonstrated here that autocrine production of hGH by mammary carcinoma cells decreased transcription of the PTGF-β gene in a p53-independent manner. Thus, we observe that autocrine production of hGH results in a similar percentage decrease in PTGF-β gene transcription in the absence of the two putative p53-binding sites in the PTGF-β promoter, and the inhibitory effect of autocrine production of hGH by MCF-hGH cells on the PTGF-β promoter was maintained when several PTGF-β gene promoter active (p53-1–143) and p53-1–273) or active (p53-1–281) mutants of p53 (16, 42) were utilized. Thus autocrine production of hGH by mammary epithelial cells will antagonize the cellular response to p53 and therefore promote inappropriate cell survival potentially leading to neoplastic transformation. p53 is an important mediator of the cellular response to DNA damage and activates genes responsible for both cell cycle arrest and apoptosis (43). How-
ever, inhibition of p53-regulated genes by autocrine production of hGH is not a general cellular response as autocrine hGH has been demonstrated previously by us to up-regulate two p53 regulated genes, namely GADD45 (24) and p21\textsuperscript{Waf1/Cip1} \(^3\). Furthermore, IGF-BP3 is both a p53- (44) and hGH-regulated (45) gene. Interestingly, there exists a STAT-binding site in the promoter region of the PTGF-\(\beta\) gene (25), and this may constitute one mechanism for the observed effect of autocrine hGH on PTGF-\(\beta\) gene transcription. STAT molecules can either stimulate or repress transcription depending on the promoter context (46, 47), and hGH has been demonstrated to utilize STATs for many of its transcriptional effects (48). Further analysis of the PTGF-\(\beta\) promoter should allow for the precise definition of the regulatory elements utilized by autocrine hGH to suppress PTGF-\(\beta\) gene transcription.

We have reported previously that autocrine production of hGH by mammary carcinoma cells results in increased entry to S-phase (3) and increased cell number (2). We observe here that forced expression of PTGF-\(\beta\) completely prevents S-phase entry by either MCF-MUT or MCF-hGH cells. Thus, decreased expression of PTGF-\(\beta\) would be required for autocrine hGH to promote cell cycle progression of mammary epithelial cells. However, exogenous application of hGH to mammary carcinoma cells (MCF-7) still results in equivalent entry to S-phase (3) but without a decrease in PTGF-\(\beta\) expression (this study). It may be that high expression of PTGF-\(\beta\) is incompatible with neoplastic proliferation, and therefore MCF-7 cells already have diminished PTGF-\(\beta\) expression below a critical threshold required for survival and cell cycle progression. This would therefore allow proliferation in response to a mitogen such as exogenously applied hGH without further decreases in the level of PTGF-\(\beta\). This is concordant with the fact that forced expression of PTGF-\(\beta\) alone results in cell cycle arrest and apoptosis of mammary carcinoma cells (this study and Ref. 25), including mammary carcinoma cells with autocrine production of hGH. It is interesting to note that autocrine hGH production by mammary carcinoma cells results in dramatically increased p21\textsuperscript{Waf1/Cip1} expression. Increased p21\textsuperscript{Waf1/Cip1} expression has been associated previously with inhibition of cell cycle progression stimulated by either PTGF-\(\beta\) (16) or TGF-\(\beta\) (49), and we also observe here that forced expression of PTGF-\(\beta\) in MCF-MUT cells results in increased p21\textsuperscript{Waf1/Cip1}. This observation is concordant with the published role of p21\textsuperscript{Waf1/Cip1} as the major mediator of p53 induced G1 arrest (50). However, other investigators (51) have also demonstrated recently that autocrine hGH GH Inhibits PTGF-\(\beta\) Gene Expression results in formation of mammary carcinoma (54). Increased expression of cyclin D1 has also been demonstrated to be sufficient for G1 progression in mammary carcinoma cells (28). Forced expression of PTGF-\(\beta\) prevented the autocrine hGH-stimulated increase in cyclin D1 observed in MCF-hGH cells. The mechanism by which PTGF-\(\beta\) prevents the autocrine hGH-stimulated increase in cyclin D1 remains to be determined. The level of cyclin D1 is predominantly regulated at the transcriptional level by rapid changes in the activity of the cyclin D1 promoter which is under complex control by multiple signaling pathways (55). We observed here that autocrine hGH production in mammary carcinoma cells results in increased transcription of the cyclin D1 gene which is specifically repressed by PTGF-\(\beta\). Although p44/42 MAP kinase has been demonstrated to be required for cyclin D1 gene expression (56), we have observed that both autocrine hGH and PTGF-\(\beta\) resulted in increased activation of p44/42 MAP kinase in mammary carcinoma cells, \(^4\) and therefore PTGF-\(\beta\)-inhibition of cyclin D1 expression is not p44/42 MAP kinase-dependent. The transcription of the cyclin D1 gene is also regulated by STAT5 (57), and STAT5 is required for GH-stimulated mitogenesis of islet \(\beta\)-cells (58). We have observed that PTGF-\(\beta\)-decreases STAT5-mediated transcription in mammary carcinoma cells, \(^5\) and whether this constitutes the mechanism of the observed PTGF-\(\beta\) decrease in cyclin D1 is under investigation. In any case PTGF-\(\beta\)-prevents autocrine hGH-stimulated mammary carcinoma cell cycle progression by inhibition of autocrine hGH-stimulated cyclin D1 expression.

In addition to blocking cell cycle progression of mammary carcinoma cells in response to autocrine production of hGH, we observed that forced expression of PTGF-\(\beta\) resulted in apoptotic cell death. Overexpression of PTGF-\(\beta\) has also been demonstrated by other investigators to result in apoptotic cell death of mammary carcinoma cells (25). In this regard it is interesting that the decreased transcription of the PTGF-\(\beta\) gene is only observed with autocrine-produced and not exogenously added hGH. Similarly, protection from apoptotic cell death is also only provided by autocrine-produced and not exogenously added hGH (3). Thus the suppression of PTGF-\(\beta\) gene transcription by autocrine hGH may be one mechanism by which autocrine hGH differentially affects mammary carcinoma cell behavior in contrast to exogenously added or "endocrine" hGH. Presumably the decreased production of PTGF-\(\beta\) will also result in the diminished transcription of pro-apoptotic genes and release of transcriptional repression of genes required for cell survival. Cell survival and cell death genes regulated by PTGF-\(\beta\) in mammary carcinoma cells remain to be identified. hGH may also directly regulate genes required for cell survival, and we have demonstrated recently that autocrine hGH increases transcription of the CHOP gene to result in survival of mammary carcinoma cells in a p38 MAP kinase-dependent manner (24). What needs to be determined is the mechanism and sequential order by which these genes are regulated by autocrine production of hGH. Detailed and sequential promoter analyses, identification of the relevant transcription factor response elements combined with the relevant dissection of upstream signaling pathways, should allow for the identification of primary versus secondary or tertiary events in the effects of autocrine hGH on mammary carcinoma cell behavior and in particular apoptotic cell death.

In conclusion, we have demonstrated that autocrine production of hGH by mammary carcinoma cells results in transcriptional repression of the PTGF-\(\beta\) gene with consequent de-

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\(^3\) R. Graichen and P. E. Lobie, unpublished observations.
\(^4\) D.-X. Liu, R. Graichen, and P. E. Lobie, unpublished observations.
creases in its protein product and accompanying cellular effects that include cell cycle arrest and apoptosis. Thus, one mechanism by which autocrine hGH promotes mammary carcinoma cell survival is by transcriptional repression of the protein effector molecules that promote cell cycle arrest and apoptosis. Such a mechanism is analogous and complementary to the ability of hGH to activate transcriptionally the protein effector molecules that stimulate cell cycle progression and cell survival (15). It remains to be determined what effects of autocrine hGH on mammary carcinoma cell behavior are mediated directly by autocrine hGH or indirectly via utilization of effector molecules, which themselves directly affect cellular function.

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Autocrine Human Growth Hormone Inhibits Placental Transforming Growth Factor-β Gene Transcription to Prevent Apoptosis and Allow Cell Cycle Progression of Human Mammary Carcinoma Cells

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