Induction of Programmed Cell Death in Kaposi’s Sarcoma Cells by Preparations of Human Chorionic Gonadotropin

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**Background:** Isolation of the first neoplastic acquired immunodeficiency syndrome-related Kaposi’s sarcoma (KS) cell line (KS Y-1) has furthered understanding of the pathogenesis of KS. Studies with KS Y-1 cells have indicated that inhibition of KS cell proliferation occurs in early pregnancy in mice and after treatment with certain commercial preparations of human chorionic gonadotropin (hCG, a pregnancy hormone purified from urine). The activity of the commercial preparations has been attributed to an hCG-associated factor(s) (HAF). While several clinical benefits of HAF are clearly evident, the basis for its anti-KS properties remains unknown. We investigated the apoptosis-inducing effects of HAF and the expression of apoptosis-related proteins in KS cells.

**Methods:** KS Y-1 and KS SLK cells were treated with clinical-grade crude preparations of hCG, recombinant hCG, or urine fractions exhibiting anti-KS activity and then examined for features of apoptosis. Levels of proteins associated with apoptosis were monitored by western blot analysis, and cell DNA content was assessed by flow cytometry. Tumors induced in mice by inoculation of KS Y-1 cells were treated with preparations of hCG, and the tumors were examined for cell morphology and also for DNA fragmentation by use of the terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick-end-labeling (TUNEL) assay.

**Results:** The HAF present in some preparations of hCG and in urine fractions has the ability to induce apoptosis in KS cells in vitro and in vivo. HAF-triggered apoptosis was preceded by increased levels of the apoptosis-related proteins c-Myc and c-Rel and cell accumulation in G0/G1 phase of the cell cycle. KS Y-1 cells transfected with a c-Myc complementary DNA showed elevated rates of apoptosis.

**Conclusion:** The anti-KS activity of HAF appears to induce apoptosis. Such activity suggests a role for HAF in pregnancy-related regulation of cell death. [J Natl Cancer Inst 1999;91:135–43]

Kaposi’s sarcoma (KS) tumors are blood vessel-rich lesions characterized by proliferation of cytokine-dependent spindle cells, activated endothelial cells, immune cells (1–3), and the presence of human herpesvirus 8 (HHV8)-infected cells (4). These lesions express basic fibroblast growth factor (a potent angiogenic factor), platelet-derived growth factor, and vascular endothelial growth factor (5), as well as interleukin 6, hepatocyte growth factor, and interleukin 8 that may collectively contribute to the abundantly vascularized appearance of these lesions (6–8). Nodular or late-stage forms of KS harbor malignant cells, which proliferate independently of cytokines (9–11) and are often clonal (12). We and others (11,12) have isolated tumorigenic cell lines (KS Y-1 and KS SLK) that are derived from nodular KS lesions, which contained rearrangement in chromosome 3 at region 3p14, the location of the transformation-related fragile histidine triad gene. Virtually all cases of KS tumors, regardless of their epidemiologic type (acquired immunodeficiency syndrome [AIDS]-related, endemic, classical, or iatrogenic), contain HHV8 DNA sequences suggesting that it is a necessary cofactor (13), and when present with human immunodeficiency virus type 1 (HIV-1) infection, both contribute to a progressive cancer.

Our initial study (10) of tumors induced by the inoculation of KS Y-1 and KS SLK cells in nude mice revealed an unanticipated pronounced inhibition of tumor development during early pregnancy. The anti-KS activity in mice was strikingly effective during the first half of the gestation period when chorionic gonadotropin-like activity in sera from mice (and humans) is maximum (the murine equivalent of human chorionic gonadotropin [hCG] has not yet been identified). Some clinical-grade crude preparations of hCG initiated a regression of tumors induced by KS Y-1 cells in nude mice and inhibited clonogenic KS Y-1 cell foci formation (10). These animal experiments spurred human trials to test local or systemic administration of hCG for KS. IntraleSIONal and systemic treatment with commercially available clinical-grade crude preparations of hCG induced significant rates of regression of both cutaneous and of the more resistant visceral KS lesions (14,15). Because commercial preparations of hCG from various sources contain variable anti-KS activity yet equivalent endocrine activity and because highly purified hCG and recombinant hCG lack anti-KS activity, this collectively suggested to us that the activity of the commercial preparations of hCG was due to a co-purified factor (or factors) that had the ability to induce regression of KS tumors. We have termed this anti-KS factor as hCG-associated factor(s) (HAF) that is present in some, but not all, commercial clinical-grade preparations of hCG and in urine fractions has the ability to induce apoptosis in KS cells.
crude preparations of hCG and that which is present in urine during pregnancy. We have shown that anti-KS activity did not reside in the hCG molecule or in one of its subunits (16). While patients were undergoing treatment for KS with preparations of hCG, we also noted an improvement in their HIV-1 load, hematopoietic parameters, and overall well-being. Furthermore, macaques with clinical simian immunodeficiency virus infection treated with preparations of hCG showed reduced viral load, weight gain, and prevention of AIDS (16). The anti-HIV-1 and anti-KS activities appear to co-separate in partial purification of HAF, suggesting that these activities may stem from the same molecule(s) (16) but are yet to be proven conclusively. Therefore, HAF may have the ability to reverse some of the effects of AIDS, in addition to its anti-KS activity. While several clinical benefits are clearly evident with treatment with preparations of hCG, the underlying basis for these improvements, including anti-KS properties of HAF, remains unknown. To investigate the anti-KS activity of HAF, we treated KS cells in vitro and KS tumors in vivo with preparations of hCG and with partially purified fractions of HAF from the urine of pregnant women. We describe apoptosis-related morphologic changes and protein expression.

Methods

Treatment of tumors induced by KS Y-1 cells with preparations of hCG. Tumors were first induced in beige nude mice (Harlem, Frederick, MD) by subcutaneous inoculation of 1 × 10⁶ KS Y-1 cells into the dorsal lateral flanks, which consistently produced 5 × 5 mm² tumors within 2 weeks. Several commercial preparations of hCG (clinical-grade crude hCG at a dose of 500 IU [0.25 IU/μg protein]) were evaluated, but a batch of Wyeth Ayerst preparations of hCG (Wyeth Ayerst Laboratories, Philadelphia, PA) that induced apoptosis of KS Y-1 cells in culture was used in all studies except where shown otherwise. Preparations of hCG or an equivalent amount of hCG buffer (i.e., equivalent concentration of salts in commercial hCG preparations) were administered daily by injection into KS lesions and adjacent tissues for 5 consecutive days. The dose of 500 IU of commercial hCG was determined as an adequate dose required for inducing regression of the tumors, and a maximal tolerated dose was not determined. Since the active principle with anti-KS activity is not totally characterized, any chance of variation in anti-KS activity among different preparations was eliminated by using only one batch of hCG with reasonable activity. Animal care was in accord with institutional guidelines. Bidirectional tumor numbers were determined before and after treatments, tumors were resected, and hematoxylin–eosin-stained sections of tumors were examined for morphology. To study the early effects of preparations of hCG-induced tumor regression, tumors were treated intralesionally with preparations of hCG (500 IU) or buffer for 2 days. The tumor sites and surrounding tissues were resected and fixed in formalin. The terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick-end-labeling (TUNEL) assay was performed by treatment of thin tissue slide sections with terminal deoxynucleotidyl transferase for in situ extension at DNA termini by incorporation of digoxigenin-deoxyuridine triphosphate (Oncor Inc., Gaithersburg, MD). Cells with high levels of DNA termini support incorporation of digoxigenin-deoxyuridine triphosphate, which was detected by peroxidase-conjugated anti-digoxigenin antibody. Also, using the same tissue fixative method, thin tissue slices of control rat gut were concurrently analyzed for apoptosis. Cells at the base and cells at the tip of rat gut villi tissue served as reference material for live and apoptotic cells, respectively.

Cells. KS Y-1 cells were isolated and established as a neoplastic cell line from a pleural effusion of an HIV-1-infected male with KS involving the lung (10), and KS SLK cells were derived from an oral KS lesion of an HIV-1-noninfected kidney transplant recipient receiving immunosuppressive drugs (11). KS SLK cells were derived from an oral KS lesion of an HIV-1-infected male with KS involving the lung (11), and KS SLK cells were derived from an oral KS lesion of an HIV-1-noninfected kidney transplant recipient receiving immunosuppressive drugs (11). Both cell lines bear endothelial markers and induce angiogenic tumors when inoculated in nude mice. AIDS-KS4 cells are early passage (passages 3–5) hyperplastic cells directly derived from human KS lesions that express tissue specific markers, proliferate in response to inflammatory cytokine stimulation and induce formation of transient KS-like lesions when inoculated in nude mice (5,6,8). AIDS-KS4 cells are considered to represent the hyperplastic spindle cells of KS lesions. AIDS-KS4 cells as well as the two cell lines described above were maintained in a gelatin (1.5%-coated flask in RPMI-1640 medium containing the following: 15% fetal bovine serum (FBS), essential and nonessential amino acids, 1% Nutridoma (Boehringer Mannheim Corp., Indianapolis, IN), penicillin G (100 U/mL), and streptomycin (100 μg/mL). These cells (KS Y-1, KS SLK, and AIDS-KS4) lack HHV8 and HIV-1 DNA (17). Human umbilical vein endothelial (H-UVE) cells and breast cancer cells (Br-483) were included as controls; their propagation has been described by American Type Culture Collection (Manassas, VA).

Partially purified HAF from urine of pregnant women. Urine from first-trimester pregnancy (40 L) was filtered, concentrated, and then desalted on a Sephadex G25 column that effectively reduced the volume to less than 500 mL (16). The protein-containing peak was then separated on a Superdex 200 column (26/60) (Pharmacia Biotech, Inc., Piscataway, NJ) in phosphate-buffered saline (PBS). Fractions were measured for total protein levels and for concentrations of heterodimeric hCG and the hCG β-core fragment (a cleaved form of hCG) by specific immunoassays. The hCG heterodimer elutes as a 70-kd molecule and the hCG β-subunit core elutes as a 10- to 25-kd peak on gel filtration (16). hCG immunoreactivity peaked between fractions 46 and 49 of pregnancy urine concentrates. The first and second peaks of anti-KS activity elute with molecular ranges of 15–30 and 2–4 kD and their respective peak positions are remote from the elution positions of hCG or hCG β-free subunit.

Treatment of cells with preparations of hCG and purified hCG (CR 127). KS Y-1, KS SLK, and Br-483 cells were seeded into T75 flasks at 20% confluency in RPMI-1640 medium containing 15% FBS and grown for 1 day. The cells were then synchronized by incubation for 24 hours in medium containing 1% FBS, pulsed for 24 hours with medium containing 10% FBS, and then incubated in medium containing 1% FBS, preparations of clinical-grade hCG, or equivalent amounts of CR 127 (100–1000 IU/mL, National Hormone and Pituitary Program and Center for Population Research, National Institutes of Health, Bethesda, MD), thyroid-stimulating hormone (TSH), a hCG-related heterodimer glycoprotein (Sigma Chemical Co., St. Louis, MO), or buffer containing the salts in preparations of hCG. The CR 127 was available in limited quantities and was used only in the preliminary experiments. To avoid cell death related to low FBS levels, H-UVE cells were cultured in RPMI-1640 medium with 10% FBS for 24 hours, then in medium containing 15% FBS for 24 hours, and finally with 10% FBS and preparations of hCG or buffer control for the indicated intervals described above. Cells were harvested for genomic DNA isolation (Stratagene, La Jolla, CA) or for monitoring protein expression.

Staining for actin and chromatin of cells treated with crude preparations of hCG, CR 127, or partially purified HAF fractions. One hundred thousand KS Y-1 cells, KS SLK or AIDS-KS4 cells, or 10⁴ H-UVE cells were seeded onto gelatinized glass chamber slides (200 mm²) and incubated for 24 hours with 500 IU/mL of preparations of hCG or buffer. The slides were fixed with formalin and serially treated with Triton X-100 (0.01%) for 10 minutes at 25°C, with 0.4 μg/mL of fluorescein isothiocyanate (FITC)-labeled Phalloidin, which binds actin (Sigma Chemical Co.) for 30 minutes, washed with PBS, and then stained DNA with propidium iodide (0.5 μg/mL) (Sigma Chemical Co.) for 15 minutes (18). The stained slides were washed and mounted with Slow Fade (Molecular Probes Inc., Eugene, OR) and examined (1000x) through a dual emission filter system. Cells were maintained in RPMI-1640 medium containing 10% FBS and grown for 24 hours with clinical-grade crude preparations of hCG and equivalent amounts of CR127, recombinant hCG, TSH, or with chromatographic fractions 48, 67, and 76 (the latter two contain anti-KS clonogenic activity) from human pregnancy urine concentrates (16), and fixed according to instructions described above, but without Triton X-100. The slides were then stained with propidium iodide and examined for chromatin and cellular morphology under confocal microscopy.

Protein expression in cells undergoing apoptosis after treatment with preparations of hCG. KS Y-1, KS SLK, and H-UVE cells were synchronized by pulsing with FBS and treated with clinical-grade crude preparations of hCG as described above. After 24 hours of incubation with preparations of hCG, the cells were washed with PBS and lysed in buffer containing 1% Nonidet and the protease inhibitors leupeptin and aprotonin. The lysate was clarified by centrifugation (11 750×g) at 4°C for 15 minutes. Clarified lysate (100 μg protein) was size separated by reducing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose. Filters were blotted with antibodies to c-Myc (9E10) (Santa Cruz Biotech, CA), retinoblastoma (RB) protein (Santa Cruz Biotech), Bcl-2 (Dakopatts, Carpenteria, CA), and c-Rel (Santa Cruz Biotech).
Oligonucleosomal DNA Cleavage and In Situ Apoptosis in KS Tumors After Treatment With Preparations of hCG

To demonstrate the effects of clinical-grade crude hCG (HAF in the preparations of hCG), KS tumors of equal size (5 × 5 mm) induced by the inoculation of KS Y-1 cells in beige nude mice were treated intrasessionally with preparations of hCG (500 IU) or buffer daily for 5 days. The buffer-treated lesions in three mice showed continued tumor progression (>5 × 5 mm), whereas three mice treated with intrasessional preparations of hCG showed near-complete tumor regression (except that acellular bands of tissue remained) confirming the anti-KS effect of preparations of hCG observed in previous studies (10). To examine the early effects of preparations of hCG on tumors, KS Y-1 and KS SLK cell-induced tumors and surrounding mouse tissues were treated with preparations of hCG (500 IU) or buffer for 2 days. On the third day the tumor sites and surrounding tissues and other control tissues were resected and genomic DNA extracted. DNA isolated from buffer-treated tumors and from endothelium-rich tissue (aorta) treated with preparations of hCG (Fig. 1) showed high-molecular-weight DNA (>23 kilobase) as determined on ethidium bromide-stained agarose gel. The DNA of tumors treated with preparations of hCG showed DNA in an oligonucleosomal fragmentation pattern typical of programmed cell death (Fig. 1). Some of the preparations of hCG-treated tumors showed minimal detectable fragmented DNA that was not easily visualized (Fig. 1, left lane; KS Y-1 with 500 IU) while the TUNEL assay showed enhanced apoptosis (see below), indicating that detection of apoptosis by genomic DNA examination was a less-sensitive technique. Thus, there was evidence that the regression of KS tumors induced by the preparations of hCG was associated with oligonucleosomal DNA fragmentation.

To analyze the early mechanism leading to tumor regression, formalin-fixed slides of tumor tissues were stained with hematoxylin–eosin for morphologic analysis. Tumors treated with preparations of hCG showed hypocellular areas (Fig. 2, A, X) with a few densely staining cells, but no inflammatory cell infiltration. In contrast, surrounding normal mouse cells (Fig. 2, A, Z) did not show depletion of cells or did not show cell morphology suggestive of an occurrence of apoptosis despite equivalent exposure of tissues to preparations of hCG. To further assess the mechanism of tumor regression induced by preparations of hCG, tumors were analyzed for the presence of DNA termini by using digoxigenin-deoxyuridine triphosphate nucleotide incorporation in TUNEL assay (Fig. 2, B, X, Z) (9). Peroxidase activity (indicative of the presence of DNA terminal) was markedly evident within residual small cells in hypocellular areas of tumors treated with preparations of hCG. Notably, there was no extracellular staining, indicating that the DNA termini generated were retained in cells or intact cell fragments, which is consistent with apoptosis and not necrosis, to account for depletion of cells. The aforementioned cell-associated high peroxidase activity and morphologic changes were not observed in mouse tissues similarly treated with preparations of hCG (Fig. 2, B, X, Z) or in buffer-treated tumors (Fig. 2, C). Specifically, the treated tumors contain areas showing on average ±standard deviation (SD) 6.5% (±1.3%), 7.3% (±2.2%), 7.5% (±1.9%), and 8.0% (±1.4%) positive staining cells per high-power field, whereas preparations of hCG-treated adjacent mouse tissues showed 1% (±0.8%), 1% (±0.8%), 0.2% (±0.5%), and 0.5% (±1%) positive cells per high-power field, indicating significantly higher apoptotic rates in treated tumors (P < .05). Buffer-treated tumors contained 2.5% (±1.3%) and 2% (±1.4%) positive cells per high-power field (Fig. 2, C). Control rat gut tissue sections concurrently stained in TUNEL assay showed the presence and absence of apoptosis indicated by peroxidase labeling in cells residing in the tip and the base of villi, respectively (Fig. 2, D). Thus, treated tumors showed significantly higher rates of apoptosis over control buffer-treated tumors. These observations account for HAF-related KS lesion regression.
DNA Fragmentation in KS Y-1 and KS SLK Cells Treated With Preparations of hCG

To determine whether the anti-KS activity of preparations of hCG is a direct effect, tumor and control cells in culture were treated with preparations of hCG (400 IU/mL) for 48 hours. Genomic DNA was isolated from cells and size-fractionated by electrophoresis. The DNA from KS Y-1, KS SLK, and AIDS-KS4 cells, after treatment with preparations of hCG, was fragmented in an oligonucleosomal pattern, whereas DNA from similarly treated control H-UVE (21) and Bt 483 cells, as well as buffer-treated KS cells, was of high molecular weight (Fig. 3). Treatment of KS Y-1 and KS SLK cells with highly purified hCG (CR 127) did not induce cell death (see below) suggesting selective HAF activity in clinical-grade crude preparations of hCG was not due to hCG.

Morphologic Changes Induced by Preparations of hCG

To characterize (with enhanced resolution) the early steps accompanying apoptosis, cells were monitored by epifluorescence microscopy 24 hours after treatment with preparations of hCG. Fig. 4 shows representative morphology of the buffer-treated cells, which contain a relatively high, cytoplasmic-to-nuclear size ratio (Fig. 4, A–D). Buffer-treated cells showed sharply defined nucleoli and well-defined filamentous actin of the cytoskeleton that was distributed widely throughout the cytoplasm. In contrast, KS Y-1, KS SLK, and AIDS-KS4 cells treated with preparations of hCG (Fig. 4, E–G) exhibited overall smaller cell size and contained small nuclei with focal areas of intense homogeneous nuclear staining. Similarly treated H-UVE cells did not show the morphologic changes found in KS Y-1 cells treated with preparations of hCG (Fig. 4, H) as observed by others (21). This acquired morphology is found in the earliest recognizable phases of apoptosis (18). In addition, cells treated with preparations of hCG displayed a low cytoplasmic-to-nuclear size ratio and a reduced content of filamentous actin that was poorly defined and preferentially retracted to the perinuclear area as observed during early apoptosis (18). Moreover, examination by confocal microscopy, revealed a twofold to fourfold reduction in the cross-sectional nuclear area and in the overall cell area of treated KS Y-1 cells compared with buffer-treated KS Y-1 cells. Thus, HAF in clinical-grade crude preparations of hCG induces prototypical early cell morphology changes of apoptosis.

To begin to identify HAF that is present in human pregnancy urine, KS Y-1 cells were selected for testing the limited available HAF material isolated in chromatography-purified fractions from urine. The KS Y-1 cells were treated with HAF fractions for 48 hours and cells labeled with propidium iodide. Commercial preparations of hCG (Fig. 5, B) and urine HAF fractions 67 (Fig. 5, E) and 76 (Fig. 5, F) induced reduction in cell size and diffuse staining and clumping of chromatin, which is consistent with apoptosis. Insufficient material in HAF fractions 67 and 76 prevented further evaluation of these fractions. In contrast, buffer treatment (Fig. 5, A), CR 127 (Fig. 5, C), and urine fraction 48 (Fig. 5, D) did not acquire morphology of apoptotic cells even with a much higher concentration (188 μg/mL), suggesting specific anti-KS activity in fractions 67 and 76. In additional experiments, treatment of KS SLK and KS Y-1 cells with CR 127, recombinant hCG, or TSH did not induce changes in cell number over 5 days, indicating lack of anti-KS activity.

Fig. 2. Tumors induced in nude mice by inoculation of Kaposi’s sarcoma (KS) Y-1 cells undergo apoptosis after treatment with preparations of human chorionic gonadotropin (hCG). Representative micrographs of sections of preparations of hCG-treated and buffer-treated KS Y-1 tumor in nude mice are shown. A) Shows hypocellular area (X) with pyknotic nuclei. These morphologic changes were not observed in unaffected mouse tissue (Z). B) The similarly treated tissues analyzed for the presence of DNA termini by terminal deoxynucleotidyl transferase-mediated digoxigenin deoxyuridine triphosphate nick-end-labeling (TUNEL) assay show some tumor cells (X) staining strongly positive with the smallest cell bodies containing more intense activity, whereas preparations of hCG-treated mouse tissues (Z) did not show activity. C) Buffer-treated tumor and mouse tissues showed much reduced rates of apoptosis by the TUNEL assay. D) For positive and negative results, rat gut in TUNEL assay shows positive and negative cells at the tip and at the base of villi, respectively. The brown immunoperoxidase reaction product indicates positive detection of significant levels of DNA termini.

Fig. 3. Preparations of human chorionic gonadotropin (hCG) induce oligonucleosomal DNA fragmentation of Kaposi’s sarcoma (KS) cells in culture. KS Y-1 and KS SLK cells, acquired immunodeficiency syndrome (AIDS)-KS and control primary cells, human umbilical vein endothelial (H-UVE) and breast cancer (Bt 483) cells were treated for 48 h with preparations of hCG 400 IU/mL (+) or buffer (−). Genomic DNA was isolated and electrophoretically separated in a 1.2 % agarose gel and stained with ethidium bromide.
Fractions 67 and 76 also abrogated formation of KS Y-1 cell foci in clonogenic assays, as shown previously (16). Fractions 67 and 76 do not contain hCG heterodimer, hCG subunits, or β-core of hCG. Thus, partial purification of HAF from urine of pregnant women contains apoptosis-inducing factor(s) that are not attributed to hCG, its subunits, or β-core of hCG.

**Protein Expression in Apoptosis Induced by Preparations of hCG**

Because preparations of hCG are known to stimulate the growth of some cells and induce apoptosis in others, we anticipate that preparations of hCG-stimulated proliferation-associated pathways in KS cells preferentially operate in these cells to accomplish programmed cell death (21–26). After treatment with clinical-grade crude preparations of hCG (400 IU/mL) for 24 hours, extracts of KS Y-1 and KS SLK cells were examined for proliferation-associated protein expression by western blot analysis. While buffer-treated cells and cells with no additional treatment contained undetectable levels of c-Myc, both KS Y-1 and KS SLK cells when treated with preparations of hCG showed markedly enhanced levels of c-Myc (Fig. 6, A).

**Fig. 4.** Preparations of human chorionic gonadotropin (hCG) induce morphologic alterations consistent with apoptosis. Cells were seeded in chamber slides and incubated with buffer or preparations of hCG for 24 hours. The cells were fixed and serially stained for actin and chromatin with fluorescein isothiocyanate-labeled Phalloidin and propidium iodide, respectively, and observed under fluorescence microscopy as outlined in the “Methods” section. The buffer-treated cells (A–D) and human umbilical vein endothelial (H-UVE) cells treated with preparations of hCG (H) contain a high cytoplasm to nuclear ratio and well-defined filamentous actin distributed widely throughout cytoplasm. In contrast, Kaposi’s sarcoma (KS) Y-1, KS SLK, and acquired immunodeficiency syndrome (AIDS)-KS4 cells treated with preparations of hCG (E–G) exhibited contraction of cytoplasm, accumulation of poorly defined filaments in the perinuclear area, and a lower cytoplasm to nucleus ratio, all changes characteristic of early apoptotic changes (18).

**Fig. 5.** Pregnancy urine-derived human chorionic gonadotropin (hCG)-associated factor (HAF), preparations of hCG (clinical-grade crude), but not highly purified hCG, induce apoptosis of Kaposi’s sarcoma (KS) Y-1 cells as visualized through confocal microscopy. A) KS Y-1 cells were treated with buffer, B) with clinical-grade preparations of hCG (Wyeth Ayerst Laboratories, Philadelphia, PA), C) CR 127, D) or partially purified HAF from urine fractions 48, E) fraction 67, or F) fraction 76, and stained with propidium iodide. Panels show normal heterogeneous DNA staining of large nuclei in cells treated with buffer, CR 127 and urine fraction 48. In contrast, cells treated with preparations of hCG, partially purified HAF from urine fractions 67 or 76 showed homogeneous DNA staining in a clump pattern in small nuclei. After incubation of cells for 24 hours with the different treatments, the cells were fixed and stained with propidium iodide and visualized under confocal microscopy as outlined in the “Methods” section.
of hCG (400 IU/mL) induced c-Rel protein expression, indicating a coordinated expression of at least two proto-oncogenes known to participate in the same signaling pathway (23) (Fig. 6, B). Additionally, KS Y-1 cell expression of RB protein did not show changes in the overall levels of RB after treatment with preparations of hCG (data not shown). However, there was a preferential reduction in the hyperphosphorylated form (higher molecular weight of the two bands) of RB in KS Y-1 cells (Fig. 6, B) after treatment with preparations of hCG. The diminished hyperphosphorylated levels of RB is consistent with accumulation of cells in G<sub>0</sub>/G<sub>1</sub> phase and this type of cell cycle regulation is characteristic of certain apoptotic pathways (27). Moreover, western blot analysis showed that treatment with preparations of hCG lowered Bcl-2 levels (by ~50%), whereas the levels of p53 remained unchanged (data not shown), as commonly noted in programmed cell death induced by the nongenotoxic type of stimuli (22). Thus, these data indicate HAF ultimately induces apoptosis following expression of proliferation-associated proteins as observed with other mitogen-triggered apoptosis.

To better determine c-Myc expression in the apoptotic process induced by preparations of hCG, we examined whether c-Myc expression in itself was sufficient to stimulate programmed cell death. To test this, KS Y-1 cells were electroporated with pLTRhmyc and cells evaluated for c-Myc expression (at 24 hours) by western blot analysis. These pools of cells were examined for cell death by following the same protocols for preparations of hCG treatment of cells. Whereas the three LTRhmyc-transfected pools contained an average (±SD) of 23 (±13.1) cells per high-power field with morphology consistent with apoptosis, the vector-alone transfected cells and mock-transfected cells contained 5.2 (±6.4) and 4 (±8.1) apoptotic cells per high-power field, respectively. Thus, induced c-Myc expression stimulates apoptosis, suggesting that c-Myc may participate in programmed cell death in KS cells after treatment with preparations of hCG.

KS Y-1 and KS SLK cells (Fig. 7, A and B, respectively) were found to express modest levels of Fas, as shown by the shift in black peak from white peak (isotype antibody control) with anti-fas antibody. We tested whether these cells were susceptible to Fas-mediated cell killing by treating cells with anti-Fas antibody (0–100 μL of CH-11) that induces apoptosis but did not observe enhanced apoptosis (<2% apoptotic cells in both antibody-treated cells and isotype control antibody-treated cells). Given these negative results, it is likely that HAF-mediated apoptosis involves mechanisms independent of Fas receptor stimulation.

In a second strategy to analyze protein expression in HAF-induced apoptosis, cells were monitored for DNA and proto-oncogene content by flow cytometry analysis. KS Y-1 cells treated with preparations of hCG and stained with propidium iodide contained a subset of cells with hypodiploid levels of DNA content (Fig. 7, D) that was absent in buffer-treated cells (Fig. 7, C). The cells with hypodiploid DNA content were of relatively smaller size as shown by the epifluorescence studies described above and findings are consistent with an apoptotic mechanism. The cells treated with preparations of hCG also preferentially accumulated in G<sub>0</sub>/G<sub>1</sub> phase (Fig. 7, D). These observations are in agreement with accumulation of hypophosphorylated RB protein (Fig. 6, B). Antibody staining of cells treated with preparations of hCG exhibited a higher number of cells expressing c-Myc (37%–78%), c-Rel (27%–48%), and unchanged levels of RB protein (Table 1). In addition, treated cells contained fewer cells (by ~50%) expressing Bcl-2. These results indicate coordinated protein expression as well as cell cycle modulation in HAF-induced programmed cell death.

**DISCUSSION**

Our initial observation linking anti-KS activity with factors associated with early pregnancy in mice (10) has led to these studies that attribute novel apoptotic HAF activity present in some clinical-grade crude preparations of hCG (which is commercially prepared from urine of pregnant women) and in urine concentrates from pregnant women prepared in our laboratory. Here, we show HAF induces KS cell programmed cell death in cell culture and in vivo as demonstrated by oligonucleosomal DNA fragmentation and the selective accumulation of abundant DNA termini in individual tumor cells in culture and in a KS tumor in an animal model. Cells treated with preparations of hCG undergo typical cellular morphologic changes that are features of cells that undergo apoptosis. This includes nuclear condensation and global cell shrinkage with dissolution of the peripheral actin cytoskeleton (18). Apoptosis follows up-regulated c-Myc and c-Rel protein levels and a simultaneous reduction of Bcl-2 levels that were observed both by western blot analysis and flow cytometry analysis. Aside from our initial studies describing the anti-KS effect of clinical-grade crude preparations of hCG, others (21) have noted KS cell apoptosis with nonclini-
Fig. 7. Kaposi’s sarcoma (KS) Y-1 and KS SLK cells express Fas and preparations of human chorionic gonadotropin (hCG) induce apoptosis by flow cytometry analysis. A) KS Y-1 cells and B) KS SLK cells express modest levels of Fas by flow cytometry analysis. The open peak indicates activity with isotype control antibody whereas the black (filled peak) indicates activity with anti-Fas antibody. Preparations of hCG treatment reduce the DNA content of KS Y-1 cells (C and D). Whereas buffer-treated cells (C) contain more than 95% of cells with at least 2N DNA complement, the preparations of hCG-treated cells (D) exhibit a substantial higher number of cells with lower DNA content. Cells treated with preparations of hCG also preferentially accumulate in G0/G1. For Fas expression, the cells were fixed and stained with anti-Fas antibody (CH-11) and subsequently with fluorescein isothiocyanate-labeled antibody as described in the ‘Methods’ section. For DNA content analysis, cells were treated with buffer or preparations of hCG for 48 hours and were fixed and incubated with propidium iodide as described in the ‘Methods’ section. The cells were then monitored for DNA content by flow cytometry analysis. For panels C and D, the vertical axis indicates the relative cell number and the horizontal axis (left to right) is the relative DNA content of propidium stained cells (low to high). Stained cells were analyzed by flow cytometry for expression of other proteins as shown in Table 1.

Table 1. Preparations of human chorionic gonadotropin (hCG) modulates proto-oncogene levels in Kaposi’s sarcoma Y-1 cells undergoing apoptosis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cells expressing protein, %</th>
<th>Buffer</th>
<th>Preparations of hCG</th>
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<tr>
<td>c-Myc</td>
<td>37</td>
<td>78</td>
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</tr>
<tr>
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<td>27</td>
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*Proto-oncogene expression in KS Y-1 cells treated with preparations of hCG were examined by flow cytometry. KS Y-1 cells were treated with preparations of hCG (400 IU/mL) or buffer for 24 hours. The cells were fixed and stained with antibodies for targeting proteins indicated and examined by flow cytometry analysis as described in the ‘Methods’ section. The results are from representative studies and indicate the percentage of positive staining cells over signal generated with control isotype antibodies.

The newly described apoptotic effect attributed to HAF resembles the apoptosis pathway best followed by a number of growth factors related to programmed cell death. In various cell systems, extracellular ligands and hormones that stimulate cell growth pathways can also, under the restricted conditions, induce apoptosis. Because preparations of hCG can induce proliferation and/or induce c-Myc in some normal and neoplastic cells (26, 29), it was anticipated to precipitate apoptosis, perhaps by diverting an activated cell proliferation pathway to a cell death end point (23–25). Here, HAF-induced c-Myc and c-Rel expression, as well as induced c-Myc expression in transfectants, was followed by KS cell apoptosis, suggesting participation of these transcription factors in HAF-induced cell death. Preparations of hCG-stimulated c-Myc and c-Rel concur with results from other laboratories showing regulation of AP-1 complex formation in HAF-induced apoptosis (28, 30). Even though c-Myc-related apoptosis has extensively been described in cell culture, a study (25) implicates c-Myc in apoptosis as a mechanism in cancer regression in vivo.

The identity of HAF is unknown. Fractions of preparations of hCG containing HAF activity are distinct from hCG, its subunits, or nick forms and is characterized as a protein by denaturing and protein digest experiments (16). Others (31) have found ribonuclease isolated from preparations of hCG to contain anti-KS activity; however, the purified fractions with HAF prepared in our laboratory lack ribonuclease. Fractions containing HAF-induced apoptosis of KS cells were not blocked by antiribonuclease antibodies. Interestingly, hCG is susceptible to proteolytic cleavage in vivo and can potentially produce peptides that activate receptors. The β subunit of hCG can be potentially cleaved to form hCGβ AA 37–57. Synthetic forms of this peptide form amphipathic α helical structures that can mimic the hCG heterodimer in activating its cognate receptor, albeit at several-fold higher concentrations (32).

Since HAF activity is found in urine in early pregnancy (16), its presence may be linked to the substantially lower rates of AIDS-KS in women versus men (33). HIV-1-infected women have lower rates of HHV8 infection and KS compared with HIV-1-infected men. Even though nonpregnant women already exhibit lower rates of KS, one would predict that pregnancy via HAF would offer enhanced protection against development of KS or HHV8 infection. However, a single retrospective study by Rabkin et al. (34) in a small number of HIV-1-infected African women found that pregnancy was not associated with lower KS rates. For other types of cancers, such as mammary cancer in humans and rats, the pregnancy state does confer subsequent lower rates of tumor occurrence. During pregnancy, breast tissue undergoes differentiation that renders breast tissue cells resistant to transformation (35). In contrast, the HAF effect we observed
with KS cells and preparations of hCG is primarily an apoptotic mechanism. Although stimulated cell differentiation can subsequently include apoptosis in some cells (36) and a transient cell differentiation effect in our studies cannot be ruled out, the overwhelming evidence suggests that HAF induces an immediate and primarily apoptotic effect. Indeed, apoptosis appears to be the basis for the demonstrated high remission rates (>80%) of intralesional preparations of hCG in a phase II trial of human KS (14) as well as high remission rates of the more advanced and resistant visceral KS in response to systemic treatments with preparations of hCG (15). The apoptosis activity of preparations of hCG indicates that a human-derived factor, HAF, is directly involved in KS cell death and holds promises for biologic-based anticancer therapy and further suggests the existence of apoptosis regulation that has previously been unappreciated.

REFERENCES


NOTES

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