

tion by disrupting the intracellular supply of components required for axonal integrity. In particular, there is a deficient transport of mitochondria into motor axons distal to the neurofilament swellings. A shortage in mitochondria might be expected to cause a severe disturbance in energy metabolism, resulting in a neuropathy. Such a 'dying back' model of nerve degeneration has been proposed as a plausible mechanism for ALS<sup>9, 11</sup>. A disruption of axonal transport by neurofilament disorganization is a pathological mechanism consistent with several aspects of ALS. First, it provides an explanation for the cellular selectivity of the disease. Large motor neurons are particularly vulnerable to neurofilament abnormalities because of their high level of synthesis of neurofilament proteins<sup>12</sup>. Second, there is a retardation in the slow axonal transport of cytoskeletal elements during ageing<sup>13</sup>, a factor that can predispose to the disease. Third, involvement of neurofilaments in ALS pathogenesis is compatible with the finding of neurofilament accumulation in motor neurons of some familial ALS cases induced by mutations in the superoxide dismutase gene *SOD-1* (cited in ref. 3), and with reports of aberrant neurofilament swellings in degenerating motor neurons of transgenic mice that express a mutant form

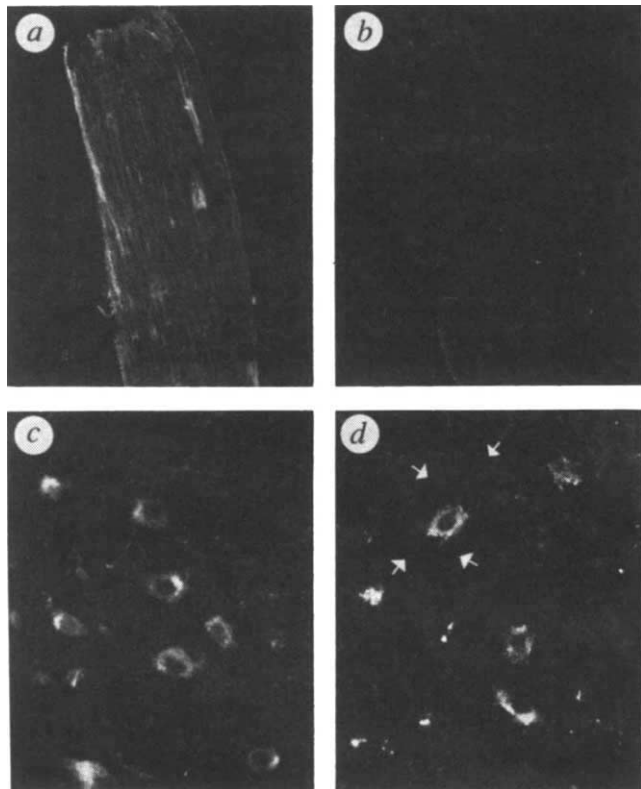


FIG. 4 Immunofluorescence detection of mitochondria in ventral roots and spinal motor neurons. The ventral root axons from an *NF-H* transgenic mouse yielded a weak anti-mitochondria staining (b) as compared with those from a normal mouse (a). Magnification  $\times 72$ . c, Mitochondria are detected throughout the cytoplasm in normal spinal motor neurons. d, In degenerating motor neurons of *NF-H* transgenics, the mitochondria, which are surrounded by large NF aggregates, are trapped near the nucleus. The arrows point to cell boundaries. c and d, Magnification  $\times 286$ .

**METHODS.** Immunofluorescence microscopic analysis. Anaesthetized mice were perfused with 0.9% NaCl and 4% paraformaldehyde in sodium phosphate buffer, pH 7.4. Sciatic nerves were rinsed in phosphate buffer and immersed in 15% sucrose and phosphate buffer. Cryostat sections (10  $\mu$ m) were first incubated with anti-inner-membrane mitochondria antibody (Serotec MOM/HG/C12) and then incubated with anti-mouse biotin (Jackson Laboratories), and fluorescein-labelled streptavidin. Sections were examined under a Nikon Labophot microscope.

of human *SOD-1*<sup>14</sup>. The discovery that abnormal neurofilament accumulations can contribute to motor neuron degeneration provides a basis for developing therapeutic approaches for ALS. Attempts to reduce the neurofilament swellings, which depend on the discovery of drugs capable of downregulating neurofilament expression, might help to slow down the devastating course of this disease. □

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## Tumorigenesis and metastasis of neoplastic Kaposi's sarcoma cell line in immunodeficient mice blocked by a human pregnancy hormone

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**KAPOSI'S sarcoma (KS) occurs more often in men than in women and HIV-1-associated KS has a high occurrence in homosexual men (over 30%). Most cultures of KS tumours yield cells with properties of hyperplastic (not malignant) endothelial cells under the control of several cytokines<sup>1–7</sup>. The role of HIV-1 may be in promoting high levels of some cytokines and providing stimulation to angiogenesis by the HIV-1 Tat protein<sup>8</sup>, which synergizes with basic fibroblast growth factor in promoting these effects<sup>9</sup>. Here we describe an immortalized AIDS-KS cell line (KS Y-1) and show that these cells produce malignant metastatic tumours in nude mice**

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TABLE 1 Inhibition of KS Y-1 growth in clonogenic *in vitro* assays**(a) Inhibition by sera of pregnant mice and humans**

Cells	Source of sera	Sera (% v/v)								
		None	1%	Early pregnancy 5%	10%	1%	Late pregnancy 5%	10%		
		Mean number of colonies								
KS Y-1	Mouse ( <i>n</i> = 3)	191	37	6	0	141	105	69		
	Human ( <i>n</i> = 4)		27	11	3	167	121	79		
		Sera from non-pregnant individuals or mice								
KS Y-1	Male (human)	193	1%	178	179					
	Non-pregnant female (human)		5%	177	170	175				
C10 MJ	Mouse	309	1%	283	299	304	298	294	310	
	Human		5%	275	279	308	300	301	297	

**(b) Inhibition of growth by hCG and the beta chain of hCG**

Cells	Treatment ( $\mu\text{g ml}^{-1}$ )					
	None	hCG $12.5 \mu\text{g ml}^{-1}$	$1 \mu\text{g ml}^{-1}$ $\beta\text{hCG}$	$10 \mu\text{g ml}^{-1}$ $\beta\text{hCG}$	$1 \mu\text{g ml}^{-1}$ $\alpha\text{hCG}$	$10 \mu\text{g ml}^{-1}$ $\alpha\text{hCG}$
KS SLK	218	15	20	0	210	183
KS Y-1	234	10	24	0	224	179
C10 MJ	288	273	291	283	280	289
PBMC	320	319	287	290	318	330

a, The KS Y-1 cell line originated from mononuclear cells isolated from 2.5 l of pleural effusion of an AIDS patient with KS involving the lungs. After the depletion of T lymphocytes, monocytes/macrophages and fibroblasts by the cytotoxicity method, using monoclonal antibodies against CD2, CD3, CD4, CD8, CD10 and CD14 membrane antigens and baby rabbit complement, the cells were cultured in the absence of exogenous growth factors in an attempt to select for transformed cells. The KS Y-1 cells have been maintained for over 100 passages and, therefore, may be appropriately designated a cell line. More extensive immunological characterization by FACScan and immunofluorescence assays showed that CD34, CD31 and endoglin were expressed by KS Y-1 (our unpublished results), suggesting an endothelial cell origin. Similar results were found in a case of classical (non-HIV-1-associated) KS, although these cells (KS SLK)<sup>10</sup> did not metastasize (our unpublished results). The growth of KS Y-1 and KS SLK was not dependent on added growth factors. KS Y-1 cells were seeded in methylcellulose (0.8%, v/v). Clonogenic assays were done in the presence of an increasing amount of sera (0%, 1%, 5% or 10% v/v) from pregnant women or mice. Colonies (>50 cells) were counted after a 10-day incubation period. The number of colonies is the mean from triplicate wells after seeding  $5 \times 10^4$  cells per culture in 0.1 ml media. The s.d. did not exceed 10% of the mean values. C10 MJ is an immortalized human adult T-cell line<sup>12</sup>. b, KS Y-1, KS SLK, human neoplastic T-cells, or normal peripheral blood PHA-stimulated T-cells ( $5 \times 10^5 \text{ ml}^{-1}$ ) were seeded in methylcellulose (0.8%, v/v). Clonogenic assays were done in the absence or presence of native hCG (hCG from Sigma (CG-10) and Wyeth Ayerst Lab (APL)),  $\beta\text{hCG}$  or  $\alpha\text{hCG}$ . Results are expressed as the mean number of colonies of triplicate wells formed after seeding with  $5 \times 10^4$  cells. s.d. was less than 10% of the mean values. C10 MJ is an immortalized human T-cell line<sup>12</sup>.

PBMC, PHA-activated normal peripheral blood mononuclear cells were stimulated with PHA-P ( $5 \mu\text{g ml}^{-1}$ ) and interleukin-2 ( $10 \text{ IU ml}^{-1}$ ) for 3 days<sup>11</sup>.

and are killed *in vitro* and *in vivo* (apparently by apoptosis) by a pregnancy hormone, the  $\beta$ -chain of human chorionic gonadotropin. Similarly, chorionic gonadotropin kills KS SLK, cells from another neoplastic cell line (established from a non-HIV-associated KS)<sup>10</sup>, as well as the hyperplastic KS cells from clinical specimens grown in short-term culture, but does not kill normal endothelial cells. These results provide evidence that KS can evolve into a malignancy and have implications for the hormonal treatment of this tumour.

Both KS Y-1 (see legend to Fig. 1 for methods) and KS SLK<sup>10</sup> have abnormal chromosomes and produce malignant tumours in immunodeficient mice (Fig. 1) in contrast to all other cultured cells from KS specimens, which grow transiently in culture and do not produce neoplastic lesions. Because KS is more frequent in males and was noted to regress in pregnancy, we hypothesized that one or more hormones might be involved. Four female and two male neonatal immunodeficient Bg-nude mice (caged together) were inoculated intraperitoneally (i.p.) and twenty female and nineteen male adult mice subcutaneously (s.c.) with KS Y-1 cells. All adult mice and the neonatal males developed malignant metastatic tumours 1 month after inoculation. However, the four female litter mates who became pregnant remained

tumour free. KS Y-1 cells were then inoculated into mice in early- and late-stage pregnancy. Mice inoculated during the early stage of pregnancy (days 1–10; average gestation is 19 days) did not develop tumours. Inoculation during the latter stages (days 13–21) generated tumours of reduced size which did not metastasize (data not shown). Typical metastatic tumours developed in all non-pregnant mice.

*In vitro* proliferation and clonogenic assays<sup>11</sup> were done using KS Y-1 cells in the presence of sera obtained from humans and mice during the early and late stages of pregnancy (Table 1a). There was a marked inhibition of growth with sera obtained during the early stages of pregnancy, minimal inhibition with sera from later stages, and no effect of sera from non-pregnant mice and humans. Also, no sera effected growth of human T cells (Table 1a) or normal human umbilical vein endothelial cells (HUVEC) (data not shown). Because human chorionic gonadotropin (hCG) levels are higher during early pregnancy, we tested the effects of purified native hCG (containing 80%  $\beta\text{hCG}$  and 20%  $\alpha\text{hCG}$ ), native  $\alpha\text{hCG}$ , and native  $\beta\text{hCG}$  on KS Y-1 growth (Table 1b).  $\alpha\text{hCG}$  had little inhibitory effect, whereas both native hCG and  $\beta\text{hCG}$  inhibited KS Y-1 cell growth and the effect was dose-dependent. Similarly, growth of

TABLE 2 *In vitro* or *in vivo* pretreatment of immunodeficient mice (B2-nude) with hCG inhibits tumorigenesis induced by KS Y-1 cells

(a) <i>In vitro</i> treatment			
	Tumour size (mm)	Angiogenesis	Metastasis
hCG source A (n = 6)	0*	0	0
hCG source B (n = 6)	0*	0	0
No HCG	17×22 to 27×30	+	+
(b) <i>In vivo</i> treatment			
	Tumour size (mm)	Angiogenesis	Metastasis
None	45×35	+	+
	41×19	+	+
	42×39	+	+
	39×37	+	+
hCG source A	0×0	NA	NA
	0×0	NA	NA
	0×0	NA	NA
	2×3	+	—
	1×2	+	—
	2×2	+	—
hCG source B	0×0	NA	NA
	0×0	NA	NA
	0×0	NA	NA
	1×1	+	—
	1×1	+	—
	2×1	+	—

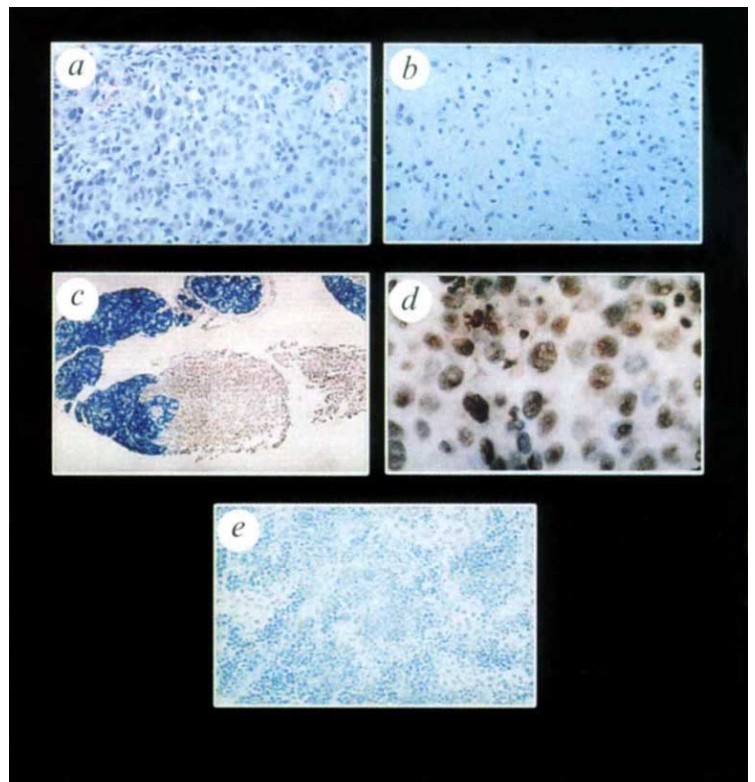
a (*in vitro*), KS Y-1 cells ( $5 \times 10^5$ ) were treated for 48 h with control (PBS) or 100 IU ml<sup>-1</sup> hCG from source A (Sigma: CG-10) or source B (Wyeth Ayerst Lab: APL) before s.c. injection into Bg-nude mice. Animals were killed 6 weeks post-inoculation. b (*in vivo*), Animals were pretreated with hCG 100 IU (i.p. or s.c.) daily for 1 week and then injected s.c. with KS Y-1,  $10^6$  cells per 0.1 ml. The mice were examined for the presence of tumours, angiogenesis and metastasis 3–4 weeks post-inoculation. Source A (Sigma: CG-10) was inoculated s.c. whereas source B (Wyeth Ayerst Lab: APL) was inoculated i.p. The experiment was repeated 5 times (with 6 mice per group). The data shown are representative samples.

n, Number of animals of test group.

\* No tumours were observed by macroscopic or light microscopic evaluation of the injection site 6 weeks post-injection.

NA, Not applicable.

FIG. 1 Tumours induced in immunodeficient mice with KS Y-1 cells undergo apoptosis when treated with hCG. a, A thin tissue section of KS Y-1 tumour (untreated) shows the presence of frequent mitotic figures. b, A thin tissue section of tumour after the mouse was treated s.c. with hCG (100 IU ml<sup>-1</sup>) daily for 7 days. There are areas with a small number of cells still remaining. Some contain dense nuclear masses. c, A metastatic tumour of pancreas in treated animals with hCG. d, Treated and e, untreated tumours of mice. c–e, The samples were stained *in situ* for the presence of cells with DNA fragmentation. Tissue slides from formalin-fixed tumours were treated with terminal deoxynucleotidase for extension of DNA ends (hydroxyl 3') and incorporation of digoxigenin-11-dUTP according to the manufacturer's instructions (Oncor, Gaithersburg, MD). Anti-digoxigenin antibody conjugated with the enzyme peroxidase allows detection of apoptotic cells that stain brown whereas viable cells stain blue.



KS SLK and cell strains KS 1510 and KS-4<sup>1</sup> was blocked in standard culture as measured by thymidine incorporation and Trypan blue exclusion, but there was little or no effect on growth of HUVEC, or human T cells (cell line C10 MJ<sup>12</sup> and phytohaemagglutinin (PHA)-stimulated peripheral blood T cells) (data not shown).

KS Y-1 cells were pretreated *in vitro* for 48 h with 100 IU ml<sup>-1</sup> (12.5 µg ml<sup>-1</sup>) hCG and then inoculated i.p. or s.c. into immunodeficient mice (Table 2). All mice inoculated with untreated cells developed metastatic tumours, whereas no tumours occurred in mice inoculated with cells pretreated with hCG. Daily administration of hCG (100 IU per day) to mice for 1 week followed by inoculation of cells markedly inhibited tumour development (Table 2).

Histological examinations of the KS Y-1 tumours show many mitotic figures (Fig. 1a). Necrosis is evident after treatment with 100 IU per day of hCG for 7 days (Fig. 1b, c). Dense nuclear masses (Fig. 1b) and DNA fragmentation assays<sup>13,14</sup> (DNA 3'-OH termini) (Fig. 1d) suggest that the effect of hCG involves cell killing by apoptosis.

It is important to know whether cells from primary KS specimens also respond to hCG. KS cells obtained from pleural effusions were grown in short-term culture. Like the immortalized KS cell lines, these cells (KS-4, KS N1512, KS N1540, KS N1544, KS N1542) were also killed by hCG (not shown). In other experiments we detected a band of *M<sub>r</sub>* 70K using radio-labelled βhCG chemically crosslinked to the cell membrane of the short-term cultured KS cells and KS Y-1. The band coincides with the *M<sub>r</sub>* of βhCG and its receptor<sup>15</sup> (Fig. 2A). As anticipated, the alpha and beta chain bands of hCG were absent when the crosslinking studies were done in the presence of 1,000-fold excess of unlabelled βhCG (Fig. 2B). In addition, all biopsy specimens from primary lesions of AIDS-KS patients (5 of 5) were positive whereas normal human skin was negative for hCG receptors by immunohistochemical staining (Fig. 2C). These results suggest that cell killing by βhCG may be due to a direct interaction with its receptor.

The low rate of KS in women might be due to the interplay of hormones in the regulation of vascular proliferation<sup>16</sup>. A



phenomenon similar to our laboratory experimental results was observed by two of us (P.H. and J.M.B.) in HIV-positive pregnant women. A 35 year old African woman with a 2-year history of AIDS-KS (observation by P.H.) (cutaneous lesions,  $CD4^+$  cells:  $61 \text{ mm}^{-3}$ ), became pregnant and 5 months into her pregnancy none of 14 KS lesions progressed, and within 5 months of delivery of a KS-free, HIV-1 seropositive child, all lesions disappeared. The second woman, a 27 year old Caucasian intravenous drug user with a 6-month history of AIDS-KS (5 KS lesions,  $CD4^+$  cells:  $490 \text{ mm}^{-3}$ ), became pregnant and within the first 2 months of pregnancy all of the observed KS lesions disappeared. Among the hormones which appear in early pregnancy,  $\beta\text{hCG}$  is the most predominant<sup>17,18</sup>.  $\beta\text{hCG}$  levels in pregnant women range from  $160 \text{ IU ml}^{-1}$  during the first trimester to under  $10 \text{ IU ml}^{-1}$  for the remainder of pregnancy<sup>19-21</sup>. hCG

belongs to the family of related hormones which include luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH)<sup>21</sup>. The hCG molecule consists of two non-covalently linked glycosylated polypeptide subunits,  $\alpha$  and  $\beta$ <sup>18</sup>. The  $\alpha$ -subunits of hCG, LH, FSH and TSH are identical, whereas the  $\beta$ -subunit of hCG is 85% homologous to  $\beta\text{LH}$  and 35% homologous to  $\beta\text{FSH}$ <sup>22,23</sup>. Though there is a functional equivalent of hCG in sera of pregnant mice<sup>24</sup>, the protein has not been identified. Our results suggest that the putative murine protein may have structural similarity to hCG. The similarity in amino-acid sequence of  $\beta\text{hCG}$  to  $\beta\text{LH}$ , a hormone also present cyclically at high levels in non-pregnant females, suggests that the cycling levels of hormones during menstruation might offer control over neovascularization<sup>16,25</sup>, and may contribute to the lower incidence of KS in women.

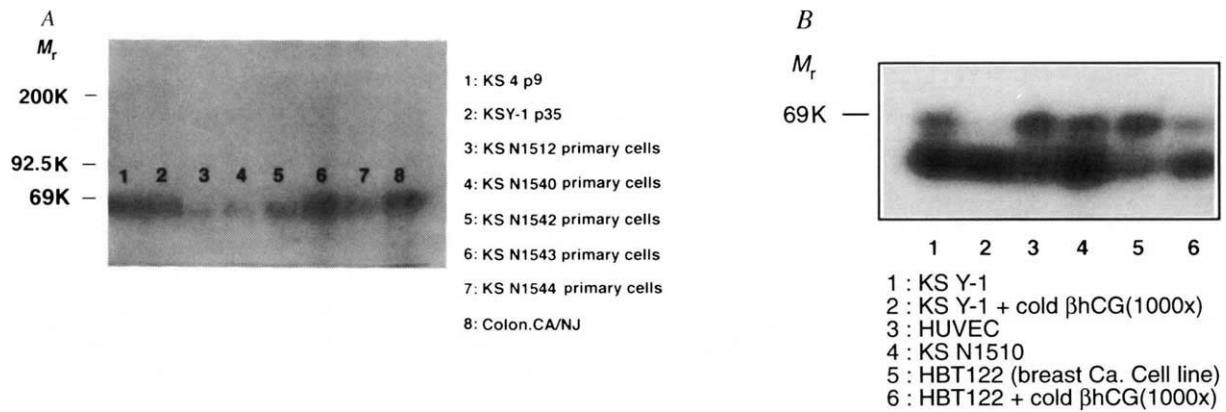
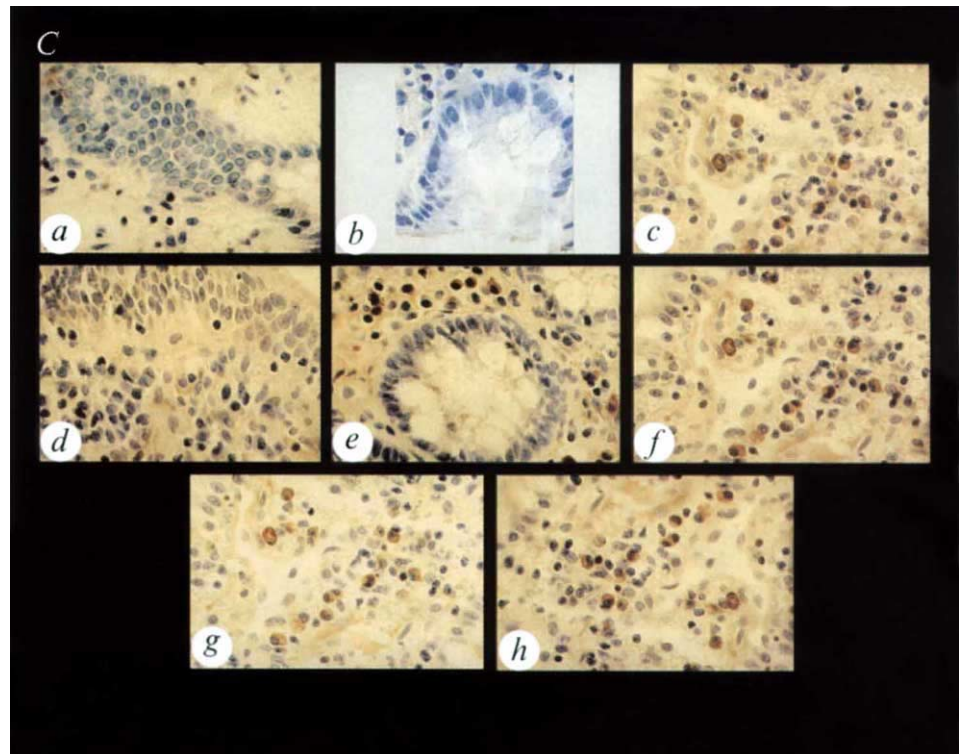


FIG. 2 A, Chemical crosslinking of hCG receptors with  $^{125}\text{I}$ - $\beta\text{hCG}$  in primary KS cells, a KS cell strain (KS-4), and the KS Y-1 cell line: lane 1, KS cell strain (KS-4, passage 9); lane 2, KS Y-1 cell line (passage 35); lanes 3-7, primary cells from AIDS-KS patients (KS N1512, KS N1540, KS N1542, KS N1543 and KS N1544); and lane 8, colon carcinoma cells (patient N1539). Binding of  $^{125}\text{I}$ - $\beta\text{hCG}$  and  $^{125}\text{I}$ -hCG were as described<sup>26</sup>. B, Binding of  $^{125}\text{I}$ -hCG was done in the absence or in the presence of 1,000-fold excess of unlabelled  $\beta\text{hCG}$ <sup>26</sup>: lane 1, KS Y-1 cell line; lane 2, KS Y-1 + a 1,000-fold excess of unlabelled  $\beta\text{hCG}$ ; lane 3, HUVEC; lane 4, KS 1510; lane 5, HBT 122 (a breast carcinoma cell line); and lane 6, HBT 122 + a 1,000-fold excess of  $\beta\text{hCG}$ . Cells ( $5 \times 10^6$ ) were incubated for 10 min at  $4^\circ\text{C}$  with  $1 \mu\text{l}$  of  $1 \text{ mg ml}^{-1}$  disuccinimidyl suberate solution (DSS) in dimethyl sulphoxide (DMSO). The reaction was stopped by quenching the unreacted DSS with  $10 \mu\text{l}$  of a  $1 \text{ mM}$  ammonium acetate solution. After 1 min the cells were washed twice at  $4^\circ\text{C}$  with  $10 \text{ mM}$  Tris-HCl,  $0.15 \text{ M}$  NaCl containing EDTA. The  $^{125}\text{I}$ -hCG pulsed, DSS crosslinked cells were extracted in  $0.5\%$  NP40 in  $0.05 \text{ M}$  Tris-HCl,  $0.15 \text{ M}$  NaCl containing  $50 \mu\text{g ml}^{-1}$  aprotinin,  $50 \mu\text{g ml}^{-1}$  leupeptin,  $0.1\%$  sodium azide and  $10 \text{ mM}$  sodium pyrophosphate at  $4^\circ\text{C}$  for 30 min. The lysates were clarified by centrifugation ( $15,000 \text{ r.p.m.}$  for 15 min at  $4^\circ\text{C}$ ) and size fractionated in a  $10\%$  SDS-PAGE gel. C, Embedded KS tissue sections from AIDS-KS patients and from normal human skin were fixed in cold acetone and double-stained with a polyclonal antibody



to hCG using a peroxidase-anti-peroxidase staining method. Non-KS skin: a, b and d (donors: a with dermatitis, and b and d, normal donors). KS skin lesions: c, e-h.

to hCG using a peroxidase-anti-peroxidase staining method. Non-KS skin: a, b and d (donors: a with dermatitis, and b and d, normal donors). KS skin lesions: c, e-h.

To our knowledge, this is the first demonstration of an anti-tumour property of  $\beta$ hCG, and offers a new strategy for treating patients with Kaposi's sarcoma. □

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## Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells

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**ARREST of cell division is a prerequisite for cells to enter a program of terminal differentiation. Mitogenesis and cytotaxis of neuronal cell precursors can be induced by the same or by different growth or trophic factors<sup>1–9</sup>. Response of PC12 cells to nerve growth factor (NGF) involves a proliferative phase that is followed by growth arrest and differentiation. Here we present evidence that the cytotaxic effect of NGF is mediated by nitric oxide (NO), a second messenger molecule with both para- and autocrine properties that can diffuse freely and act within a restricted volume<sup>10–14</sup>. We show that NGF induces different forms of nitric oxide synthase (NOS) in neuronal cells, that nitric oxide (NO) acts as a cytotaxic agent in these cells, that inhibition of NOS leads to reversal of NGF-induced cytotaxis and thereby prevents full differentiation, and that capacity of a mutant cell line to differentiate can be rescued by exogenous NO. We suggest that induction of NOS is an important step in the commitment of neuronal precursors and that NOS serves as a growth arrest gene, initiating the switch to cytotaxis during differentiation.**

When PC12 cells were tested for the diaphorase cytochemical reaction, no staining was observed. But after treatment with NGF, the cells gradually acquired an intense NADPH-depen-

dent blue colour after diaphorase staining, indicating that NOS accumulates in PC12 cells in response to NGF treatment (Fig. 1A). This increase in staining was specific for NGF and did not appear after addition of fetal calf serum or epidermal growth factor (Fig. 1A). The NGF-treated cells that were first to undergo initial morphological changes characteristic of the differentiated phenotype were also the first to show bright blue

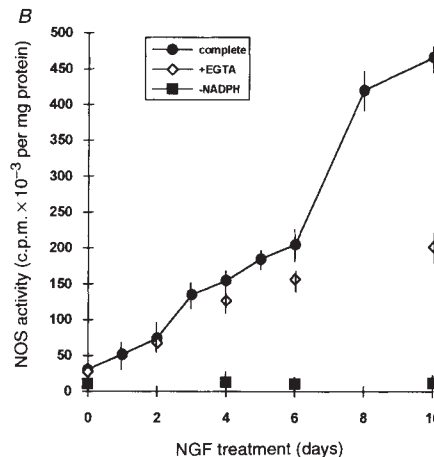


FIG. 1 NGF treatment induces NOS activity in PC12 cells. A, Diaphorase staining is induced by NGF treatment. PC12 cells were plated at low density ( $5 \times 10^4$  cells per ml) on collagen and poly-L-lysine-treated plates and NGF ( $50 \text{ ng ml}^{-1}$ ) or EGF ( $25 \text{ ng ml}^{-1}$ ) was added. At the indicated times cells were fixed with glutaraldehyde and tested for diaphorase staining. Scale bar,  $25 \mu\text{m}$ . B, Arginine-citrulline converting activity is induced by NGF treatment. Cell extracts were prepared after NGF treatment and NOS activity was determined by conversion of  $^3\text{H}$ -arginine to citrulline and NO in the presence or absence of EGTA and NADPH<sup>23</sup>. NOS activity is expressed as c.p.m. of  $^3\text{H}$ -citrulline produced per mg of protein in 30 min. Error bars represent the standard error of the mean (s.e.m.). C, Immunostaining with anti-NOS antibodies. a, d, Anti-neuronal NOS monoclonal antibodies; b, e, anti-macrophage NOS monoclonal antibodies; c, f, anti-endothelial NOS monoclonal antibodies. Cells were fixed before (a–c) or after (d–f) 9 days of NGF treatment and processed for immunofluorescence using monoclonal antibodies. The exposure time for the photomicrographs of untreated cells was  $\sim 10$  times longer than for NGF-treated cells; this length of exposure time was necessary to visualize the low level of immunofluorescent signal from the untreated cells. Note the differences in subcellular distribution of different NOS isoforms. Scale bar,  $25 \mu\text{m}$ .

METHODS. PC12 cells were an early passage stock obtained from E. Ziff's laboratory (where they were provided by L. Greene). Cells were grown on collagen-coated plates in DMEM supplemented with 5% calf serum and 10% horse serum (HyClone). For diaphorase staining, cells were fixed with 0.2% glutaraldehyde, washed in PBS and tested for diaphorase staining with 2 mM NADPH, 0.02% tetrazolium blue and 0.3% Triton X-100 as described<sup>25,26</sup>. Control experiments demonstrated that this reaction was NADPH-dependent. Diaphorase staining was not induced when cells received calf, horse, or fetal calf serum. For the  $^3\text{H}$ -arginine-citrulline conversion assay,  $0.5 \mu\text{Ci}$  of  $^3\text{H}$ -arginine was incubated with  $100 \mu\text{g}$  of cell extract together with  $0.45 \text{ mM CaCl}_2$ ,  $2 \text{ mM NADPH}$ ,  $500 \mu\text{M}$  arginine,  $10 \mu\text{g ml}^{-1}$  calmodulin and  $50 \text{ mM HEPES}$ , pH 7.5, in a total volume of  $50 \mu\text{l}$  for 30 min at  $37^\circ\text{C}$  as described<sup>27</sup>. Citrulline was separated from arginine on Dowex AG50 columns and the radioactivity in the flow-through (citrulline-containing fraction) was determined. For determination of  $\text{Ca}^{2+}$ -independent activity, the reaction was done in the presence of  $3 \text{ mM EGTA}$ . Control determinations of the enzyme activity were done in the presence of  $500 \mu\text{M L-NAME}$ , or  $20 \mu\text{l}$  of heat-treated extract, or in the absence of NADPH. For immunochemical analysis, cells were fixed with 0.5% paraformaldehyde for 5 min at room temperature followed by acetone for 15 min at  $-20^\circ\text{C}$ . The expression of various forms of NOS was visualized using anti-NOS monoclonal antibodies (Transduction Laboratories), biotinylated sheep anti-mouse antibodies (Amersham), and streptavidin-Texas red complex (Amersham). Control experiments with non-NON-specific antibodies and with omitted first antibodies gave negative results (not shown).

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