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SHORT REPORT

Insulin-like growth factor binding protein-6 activates programmed cell death in non-small cell lung cancer cells

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Insulin-like growth factor binding proteins (IGFBPs) are secreted into the extra-cellular matrix and inhibit cell growth through IGF-dependent and -independent mechanisms. In this study, we investigated the role of IGFBP-6, a relatively unexplored member of the IGFBP family, in the proliferation of non-small cell lung cancer (NSCLC) cells. Infection of NSCLC cell lines *in vitro* with an adenovirus expressing human IGFBP-6 under the control of a CMV promoter (Ad5CMV-BP6) reduced NSCLC cell number through activation of programmed cell death, as shown by cell staining with Hoechst 33342 or DNA end-labeling with bromodeoxyuridine triphosphate. The growth regulatory effect of IGFBP-6 was investigated *in vivo* by intratumoral injection of Ad5CMV-BP6 in NSCLC xenografts established in nu/nu mice. A single injection of Ad5CMV-BP6 reduced the size of NSCLC xenografts by 45%. These findings indicate that IGFBP-6 is a potent inducer of programmed cell death in cancer cells and support investigations into IGFBP-6 as a potential target in cancer therapeutics. *Oncogene* (2000) **19,** 4432–4436.

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Insulin-like growth factors (IGFs) are mitogenic peptides in a variety of cells including lung cancer cells (Macaulay, 1992). IGF-I and -II are expressed in non-small cell lung cancer (NSCLC) cell lines, and treatment of NSCLC cells with exogenous IGFs is growth stimulatory (Havemann *et al*., 1990; Quinn *et al*., 1996; Siegfried *et al*., 1992). IGFs I and II both bind to the IGF type I receptor, a tetrameric $\alpha_2\beta_2$ complex, and IGF-II binds to the IGF type II receptor, a single chain monomer. The IGF type I receptor phosphorylates the insulin receptor substrate-1, which activates signaling through mitogen-activated protein kinases, mediating the effect of IGFs on cellular growth and differentiation (Kaliman *et al*., 1998; Milasincic *et al*., 1996; Porras *et al*., 1998; Valverde *et al*., 1998). In addition to inducing cellular proliferation, signaling through the type I receptor is necessary for the survival of a variety of cancer cells (Dunn *et al*., 1998; Hongo *et al*., 1998; Lee *et al*., 1996; Lui *et al*., 1998; O'Connor *et al*., 1997; Scotlandi *et al*., 1998). For example, NSCLC cells undergo apoptosis following transfection of constructs containing either antisense IGF type I receptor or a truncated, dominant negative IGF type I receptor (Lee *et al*., 1996).

The insulin-like growth factor binding protein (IGFBP) family is a group of six distinct gene products (IGFBPs 1–6) that are secreted into the extra-cellular matrix and bind to IGFs I and II with high affinity and specificity, reducing the bioavailability of IGFs (Collett-Solberg and Cohen, 1996). A closely related family of genes termed IGFBP-related proteins (IGFBP-rP), including mac25, CTGF, *cyr*61, and *nov*, has recently been cloned that binds to IGFs with relatively low affinity and shares the NH_2-terminal cysteine-rich region of IGFBPs 1–6 but has no homology to their COOH-terminal regions (Kim *et al*., 1997). With the exception of IGFBP-6 which binds to IGF-II with higher affinity than it does to IGF-I by two orders of magnitude, IGFBPs bind to IGF-I with greater affinity (Collett-Solberg and Cohen, 1996). These biochemical properties are thought to contribute to the growth inhibitory effects of specific IGFBPs. In addition to their IGF-dependent effects, IGFBPs act through IGF-independent mechanisms. Supporting this theory, recombinant IGFBP-3 inhibits the growth of an IGF receptor-negative murine fibroblast cell line (Valentinis *et al*., 1995).

Because IGFs I and II are expressed in NSCLC cells and have a mitogenic effect on these cells, we hypothesized that IGFBPs inhibit NSCLC cell growth. In this study, we investigated the growth regulatory effects of IGFBP-6, which, of the IGFBP family members, is the least well defined in terms of its capacity to regulate cell growth. To do this, NSCLC cell lines that express low endogenous IGFBP-6 levels (A549, 226B, H596, and H226Br) were infected with an adenoviral vector that expresses IGFBP-6 under the control of a CMV promoter (Ad5CMV-BP6). Ad5CMV-based vectors infected these cell lines with >90% efficiency, as shown by analysis of cells infected with Ad5CMV-GFP, which expressed green fluorescence protein (data not shown). Ad5CMV-BP6 infection increased IGFBP-6 expression in a viral dose-dependent manner (Figure 1). The effect of IGFBP-6 on cell growth was examined by 3-(4,5-dimethylthiazol-

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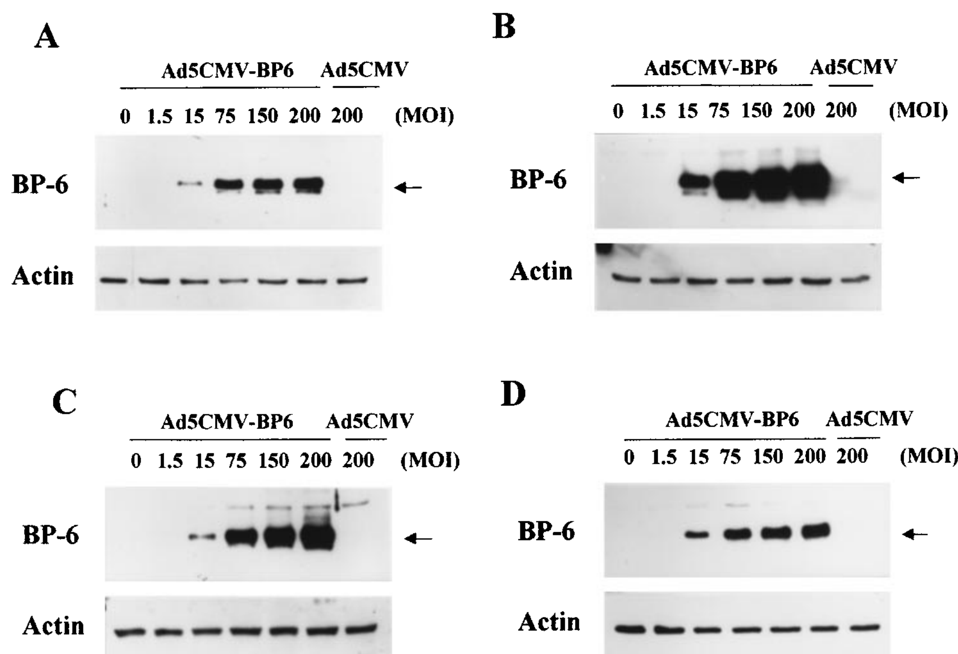

Figure 1 Ad5CMV-BP6 infection increased IGFBP-6 expression in NSCLC cell lines. Ad5CMV-BP6 was constructed by inserting the IGFBP-6 expression cassette containing the human CMV promoter, full-length human IGFBP-6 cDNA (a gift from Dr Shunichi Shimasaki, University of California-San Diego) (Shimasaki *et al*., 1991), and bovine growth hormone polyadenylation signal between *Hind*III and *Xho*I of pDE1/CMV (Fang *et al*., 1997). The IGFBP-6 shuttle vector and the recombinant plasmid pJM17 were co-transfected into 293 cells by calcium phosphate precipitation. After the cytopathic effect was detected, plaques were screened for newly-generated IGFBP-6 recombinant adenovirus (Ad5CMV-BP6) by PCR analysis of the IGFBP-6 cDNA. The presence of wild type IGFBP-6 cDNA in the recombinant adenovirus was confirmed by dideoxy-DNA sequencing. Viral titers were determined by plaque assays as previously described (Graham and Preven, 1991). The NSCLC cell lines A549 (**a**), 226B (**b**), H596 (**c**), and H226Br (**d**) were infected with the indicated titers (multiples of infection (MOI) expressed as plaque forming units (PFU) per cell) of Ad5CMV-BP6 or the parental vector Ad5CMV, and whole cell lysates were prepared 48 h later. Lysates were electrophoresed using 12% SDS–PAGE and transferred onto a BA-S-83-reinforced nitrocellulose membrane (Schleicher & Schuell, Inc., NH, USA). Membranes were incubated with polyclonal anti-human IGFBP-6 antibody (Austral Biotechnologies, San Ramon, CA, USA), or goat polyclonal anti-human actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunodetection was performed with the ECL kit (Amersham Corp., Arlington Heights, IL, USA) and diaminobenzidine

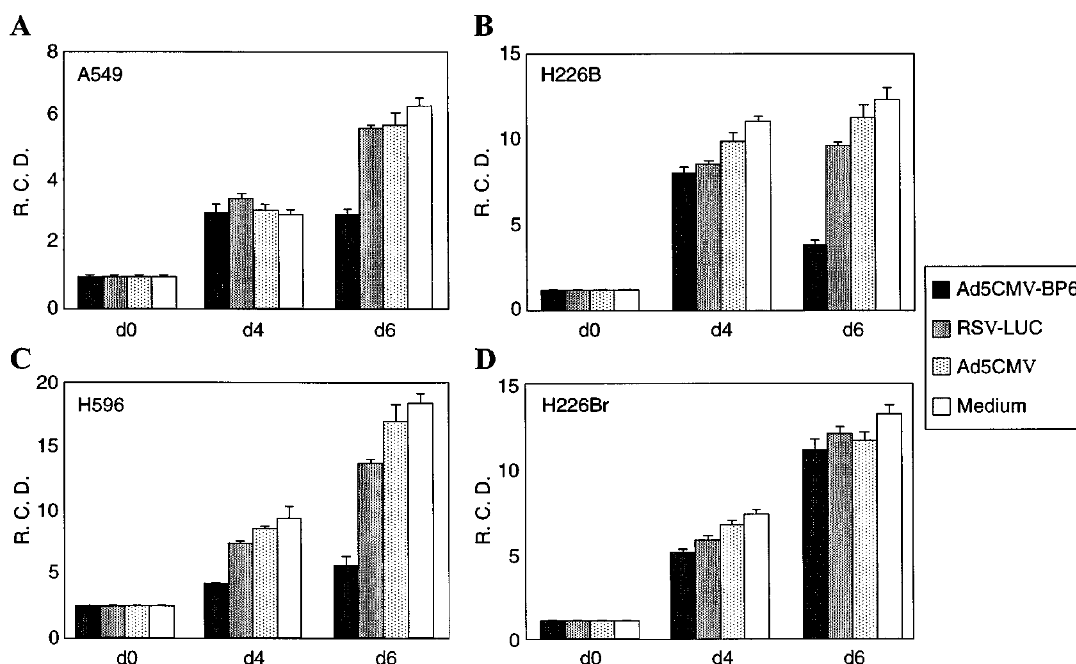

Figure 2 Ad5CMV-BP6 infection inhibited the growth of NSCLC cell lines. The indicated NSCLC cell lines were seeded onto 6-well plates in complete medium (10% FCS), infected with the indicated adenoviral vectors (MOI 50 PFU/cell) or treated with medium alone, and subjected to MTT assays the indicated number of days (d) after infection. Briefly, cells were treated with MTT, dissolved in DMSO, and formazan crystal formation was quantitated at wavelengths of 550 and 630 nm as described (Carmichael *et al*., 1987) on an EL_x808 microplate analyser (Bio-Tek Instruments). Results were expressed relative to the cell density at day 0, which is the day of infection. Each value is the mean ($\pm$s.d.) from five identical wells

2-yl)-2,5-diphenyltetrazolium (MTT) assays of cells infected with Ad5CMV-BP6, Ad5CMV (empty vector), or an adenovirus that expresses luciferase under the control of a RSV promoter (RSV-LUC) as an additional control. Relative to the effect of controls, Ad5CMV-BP6 infection prominently inhibited the growth of A549, H226B, and H596 cells in a time-dependent manner (Figure 2). In contrast, the growth of H226Br cells was minimally altered (Figure 2). H226Br cells are a variant of H226B cells and were isolated from a brain metastasis in nu/nu mice injected intravenously with H226B cells (Hwang *et al*., 1995). Relative to H226B, H226Br cells are more tumorigenic in nu/nu mice (Hwang *et al*., 1995). The levels of IGFBP-6 induced by Ad5CMV-BP6 infection were lower in H226Br cells than in H226B (Figure 1), which could have contributed to the reduced anti-proliferative effect observed in H226Br. An alternative possibility is that during clonal evolution to H226Br, H226B cells acquired somatic mutations that induced *de novo* resistance to IGFBP-6.

We investigated the mechanism by which IGFBP-6 inhibited NSCLC cell growth. Evidence for programmed cell death following Ad5CMV-BP6 infection was examined by staining cells with Hoechst 33342, a benzimadole dye that incorporates into DNA, revealing the fragmented nuclei of cells undergoing apoptosis, and by flow cytometric analysis of cells after terminal deoxynucleotidyl transferase (TdT)-induced incorporation of bromodeoxyuridine triphosphate (Br-dUTP). Hoechst staining of A549 cells and H226B cells revealed that, relative to the effect of Ad5CMV or RSV-LUC, Ad5CMV-BP6 infection markedly increased the percentage of fragmented cells, with 28.4 and 79.5%, respectively, of cells demonstrating nuclear fragmentation (Figure 3a). Flow cytometric analysis of A549 cells revealed that Ad5CMV-BP6 infection induced a comparable increase in BrdUTP incorporation, with 26.2% of the cells staining positively (Figure 3b). These findings indicate that IGFBP-6 is a potent inducer of programmed cell death in NSCLC cell lines.

We sought to investigate the growth regulatory effects of IGFBP-6 in an *in vivo* setting. H226B cells were established as xenograft tumors by injection into the dorsal subcutaneous region of nu/nu mice. Once the tumors reached a volume of at least 75 mm^3, they were injected with a single dose (10^{10} virus particles) of Ad5CMV-BP6, Ad5CMV, or an equal volume of phosphate buffered saline, and tumor size was measured every 3 days (Figure 4). Relative to the effect of Ad5CMV, Ad5CMV-BP6 injection significantly reduced tumor volume at day 12 (50% reduction, $P=0.03$) and day 15 (45% reduction, $P=0.05$) (Wilcoxon rank-sum test).

These findings add to a growing body of evidence that IGFBPs have anti-proliferative effects in a variety of cancer cells. In breast cancer cells, treatment with TGF-β2 and the anti-estrogen ICI 182780 increase the expression if IGFBP-3 and IGFBP-5, respectively, and anti-sense oligonucleotides to IGFBP-3 and IGFBP-5 abrogate the growth inhibition by TGF-β2 and ICI 182780, respectively (Gucev *et al*., 1996; Huynh *et al*., 1996). Treatment with recombinant IGFBP-3 peptide induces apoptosis in prostate cancer cells (Rajah *et al*., 1997). IGFBP-6 expression is increased in neuroblastoma cells in response to a variety of growth inhibitory stimuli, and neuroblastoma cells stably transfected with an IGFBP-6 expression vector exhibit reduced growth tumorigenicity (Grellier *et al*., 1998). IGFBP-3 expression is increased directly by p53 through a p53-responsive element in the IGFBP-3 gene promoter, suggesting that IGFBP-3 is a downstream mediator of wildtype p53, a crucial regulator of cellular proliferation (Buckbinder *et al*., 1995). Recent epidemiologic studies demonstrated that increased plasma levels of IGFBP-3 are associated with a reduced risk for lung and prostate cancer (Chan *et al*., 1998; Yu *et al*., 1999), supporting a role for IGFBP-3 as a tumor suppressor. Altogether, these findings indicate that strategies to target specific IGFBPs may be effective in cancer therapeutics.

IGFBPs inhibit cell growth through multiple mechanisms. By binding to IGFs, IGFBPs can inhibit activation of the IGF type I receptor. In addition, there is evidence that IGFBPs -3, -5, and mac25 also act through IGF receptor-independent mechanisms

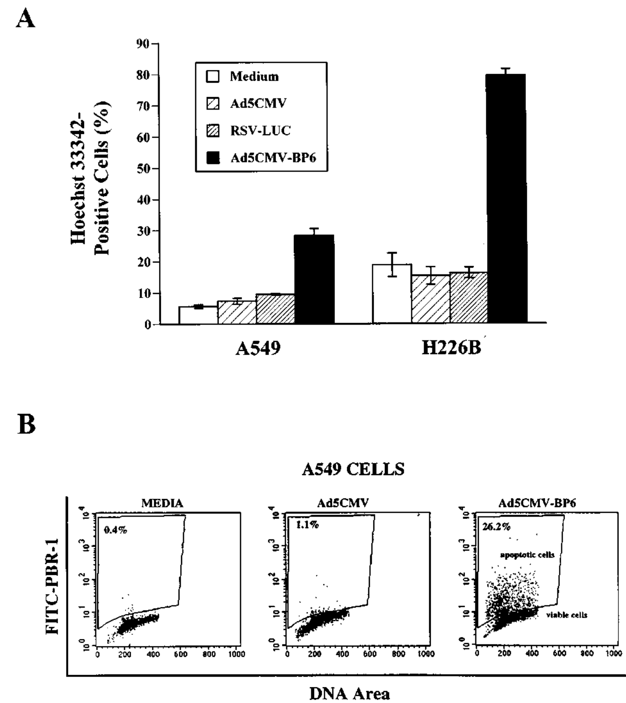

Figure 3 Ad5CMV-BP6 infection induced programmed cell death in NSCLC cell lines. NSCLC cell lines were infected with adenoviral vectors (MOI 50 PFU/cell) for 72 h, stained with Hoechst 33342 (10 μM), and analysed by non-confocal fluorescence microscopy with excitation at 360 nm (**a**) (Belloc *et al*., 1994). Cells with fragmented nuclei were identified as apoptotic cells. The percentages of apoptotic cells were determined from 1000 total cells. The experiment was repeated twice, and results represent the mean ($\pm$s.d.) percentage of apoptotic cells from the three experiments. To quantitate apoptotic cells by *in situ* DNA end-labeling, A549 cells were infected with Ad5CMV or Ad5CMV-BP6 (MOI 50 PFU/cell) for 72 h and then apoptosis was measured using the APO-BRDU staining kit (Phoenix Flow Systems, San Diego, CA, USA), which quantitates DNA breaks by TdT-induced incorporation of Br-dUTP into the 3′-OH ends of DNA strands. (**b**) Briefly, floating and adherent cells were isolated and fixed with 1% paraformaldehyde followed by 70% ethanol as indicated by the manufacturers. Cells were analysed by a FACSCAN flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with a 488 nm argon laser and CellQuest software. A dual display of DNA area (linear red fluorescence) and Br-dUTP incorporation (FITC-PRB-1) was used to determine the percentage of cells in the population that are apoptotic

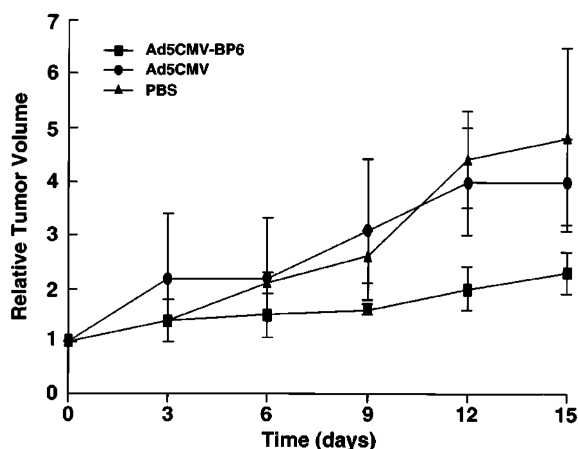

Figure 4 The growth of NSCLC xenografts was inhibited by a single intratumoral injection of Ad5CMV-BP6. H226B cells (10^7) were injected into the dorsal flank of nu/nu mice (Harlan). Once tumors measured at least 75 mm^3 in volume, 10^{10} particles of the indicated viruses or an equal amount of phosphate buffered saline (PBS) were administered in a single intratumoral injection. Tumors were measured every 3 days, and results were expressed as the mean ($\pm$s.d.) tumor volume (calculated from five mice treated identically) relative to tumor volume at day 0, which was the time of adenoviral injection

(Collett-Solberg and Cohen, 1996). For example, apoptosis is induced by recombinant IGFBP-3 in an IGF receptor-negative murine fibroblast cell line (Valentinis *et al*., 1995). This effect of IGFBP-3 is probably mediated by other cell surface receptors; recently, the type V TGF-β receptor was demonstrated to bind to IGFBP-3, potentially functioning as an IGFBP-3 receptor (Leal *et al*., 1997). Western analysis of the four NSCLC cell lines examined in this study revealed similar expression levels of IGF-I receptor, and the IGF-I receptor immunoreactive bands were of the appropriate size (data not shown), indicating that there was no obvious truncation or deletion of the receptor. Thus, we have no evidence that IGFBP-6 exerts its effect in the absence of a functional IGF-I receptor in these cell lines. However, treatment with IGF-I or -II recombinant peptide did not alter the growth inhibitory effects of Ad5CMV-BP6 on NSCLC cells (data not shown), which supports a role for IGF receptor-independent mechanisms in IGFBP-6 actions. Further, treatment with recombinant IGFBP-6 peptide (Diagnostic Systems Laboratories, Webster, TX, USA) did not inhibit NSCLC cell growth (data not shown), which is evidence that modulation of IGF-I and -II receptor activity, the primary function of secreted IGFBP-6, is not sufficient to induce cell death. Moreover, these findings suggest that Ad5CMV-BP6 induced its potent anti-proliferative effect through an intracellular form of IGFBP-6 that acts through an IGF receptor-independent mechanism.

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