Cytotoxicity of a recombinant fusion protein of adenovirus early region 4 open reading frame 4 (E4orf4) and human epidermal growth factor on p53-deficient tumor cells

Dong-Mei Wang, Yu Zhou, Hai-Jun Xie, Xiao-Li Ma, Xin Wang, Hong Chen and Bing-Ren Huang

Adenovirus early region 4 open reading frame 4 (E4orf4) protein is a novel cell death factor that selectively induces p53-independent apoptosis in cancer cells, but not in normal human cells. This study presents an approach for inhibiting p53-deficient tumor cell growth by using protein-based E4orf4 that had been genetically fused to epidermal growth factor (EGF) to ensure selective targeting of EGF receptor-overexpressing tumor cells. EGF–E4orf4 enables binding onto the cell surface and is then internalized into Saos-2 cells. The success of the process had been demonstrated by immunofluorescence assay and confocal laser microscopy. After prolonged exposure, E4orf4 remained mostly in the nuclei. EGF–E4orf4 treatment of Saos-2 cells showed dose-dependent cytotoxicity. Nearly 50% of the Saos-2 cells were killed at a concentration of 250 nmol/l. In contrast, EGF–E4orf4 showed no significant inhibitory effect iresn primary cells of human umbilical vein endothelial cells. To confirm the ability of EGF–E4orf4 to induce apoptosis, DNA fragmentation was detected using BrdUTP end-labeling. Flow cytometric analysis revealed a significant increase of apoptotic cells in Saos-2 cells treated with EGF–E4orf4, but not in the case of cells cultured in plain medium ($t=0.028$, $P<0.05$). In conclusion, these preliminary results indicate that EGF–E4orf4 could show promise as a new reagent that is more efficient and less toxic in anti-cancer therapy. *Anti-Cancer Drugs* 17:527–537 © 2006 Lippincott Williams & Wilkins.

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National Laboratory of Medical Molecular Biology, Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

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Correspondence to H. Chen, Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, No. 5 Dong Dan San Tiao, Beijing 100005, China.
Tel: + 86 10 65296406; fax: +86 10 65228797;
e-mail: hchen@public.bta.net.cn

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Introduction

Human adenovirus early region 4 open reading frame 4 (E4orf4) protein is an early viral gene product of Ad2 or Ad5. Although its function has yet to be clearly defined, it has been considered a remarkable novel cytotoxin because E4orf4 induces p53-independent apoptosis in cancer cells and its pro-apoptotic activity is higher in transformed cells than in normal cells [1–3]. E4orf4 is a small polypeptide consisting of 114 residues. It shares no extensive sequence homology with any known protein [4]. Marcellus *et al.*, using various Ad5 E4 mutants to perform luciferase and colony-formation assays, have shown that E4orf4 is the only E4 product capable of inducing p53-independent cell death when expressed alone [5]. The activity of E4orf4 on cell killing and growth inhibition was shown to depend on its ability to bind to the regulatory subunit Bα of PP2A and recruit PP2A phosphatase activity [6–9]. E4orf4 was also found to induce a Src-mediated cytoplasmic apoptotic signal, which rapidly leads to caspase-independent membrane blebbing and cell death [10–12]. Champagne *et al.* recently showed that Both Src and PP2A enzymes are critical targets of E4orf4 that may perhaps work jointly to trigger E4orf4-induced tumor cell killing [13].

Based on its ability to selectively kill cancer cells, E4orf4 could be promising in cancer therapy. All the works mentioned above have used the same method, i.e. the so-called gene therapy model, which starts by constructing a plasmid vector containing E4orf4. They then perform transfection to achieve expression of E4orf4 protein in the cells. In our study, we designed a novel recombinant fusion protein consisting of human epidermal growth factor (hEGF) and E4orf4. The EGF receptor (EGFR) is present not only on the surface of normal cells, but also on many tumor cells, especially solid tumor cells [14]. Moreover, the EGFR expression level is usually extraordinarily high in the later stages of cancer. The aim of E4orf4–EGF fusion is objective targeting via EGFR that is followed by transduction of E4orf4 into the tumor cells.

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We have previously described the genetic construction of a vector that contained the fused fragments of EGF and E4orf4. Recombinant plasmid was transformed into *Pichia pastoris* strain GS115 in which the fusion protein was expressed. The biological activities of the fused EGF–E4orf4 protein in two cell lines, MDA-MB-231 and BT325, were also demonstrated [15]. High numbers of human cancers are p53 deficient and conventional therapy for these types of cancer was found to have a poor prognosis. Hence, it will be highly beneficial to discover new reagents for p53-deficient tumors. In this study we provide some evidence for fusion protein EGF–E4orf4 as a potent anti-tumor protein reagent on p53-deficient tumor cells.

Materials and methods

Cells and culture condition

The human osteosarcoma cell line Saos-2 (p53-deficient, EGFR-positive) was maintained in DMEM supplemented with 10% FBS, 10 U/ml penicillin and 0.1 mg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were cultured in RPMI 1640 supplemented with 0.1 mg/ml heparin, 0.05 mg/ml endothelial cell growth supplement and 10% FBS. Both cell lines were supplied from the Cell Culture Center (Peking Union Medical College, Beijing, China).

Immunocytochemistry

The preparation of the recombinant fusion protein of EGF–E4orf4 has been previously described in detail [15]. Adherent Saos-2 cells were digested into a single-cell suspension and plated on pre-treated culture slides in six-well plates. After a 48-h incubation, 0.5 μmol/l EGF–E4orf4 was added and incubated for another 5 min and 24 h, respectively. At the end of the indicated time, cells were washed with PBS, fixed in 4% paraformaldehyde and then permeabilized in 5% BSA/0.1% Triton X-100/PBS for 30 min. To detect the EGF–E4orf4 protein, cells were incubated with a mixture of a monoclonal anti-hEGF antibody (Chemicon, Temecula, California, USA and a rabbit polyclonal anti-E4orf4 antiserum (both in 1:200 dilutions) for 1 h at room temperature. After rinsing in PBS, cells were incubated with biotin-labeled goat anti-rabbit IgG at a dilution of 1:200 in PBS. Cells were washed again with PBS and incubated with a mixture of a goat anti-mouse IgG antibody conjugated to FITC (1:100 dilution) and avidin-Cy3 (1:500 dilution). Cells were finally washed with PBS and mounted with Citifluor (Chem Lab, Canterbury, UK) containing 0.01% DAPI for counterstaining. The slides were viewed with an Olympus FV1000 confocal microscope. In order to prove the specificity of the fluorescent signals, the control cells were set in cells which were treated with EGF–E4orf4 for 5 min and then without a first layer of specific antibodies against EGF and E4orf4, cells were directly incubated with the second and third antibodies conjugated with either FITC or biotin followed by avidin-Cy3. Cells were also processed for immunocytochemistry using a monoclonal anti-EGFR antibody (Chemicon), biotin-labeled horse anti-mouse IgG and avidin–FITC for EGFR. E4orf4 was detected using a rabbit polyclonal anti-E4orf4 antiserum and TRITC-conjugated anti-rabbit IgG, as described above.

To observe the morphology of cell nuclei, Saos-2 cells were seeded as described above. After a 72-h incubation with EGF–E4orf4, cells were washed with PBS, fixed with 4% paraformaldehyde and mounted with Citifluor containing 0.01% DAPI. The fluorescent cells were visualized with an Olympus BX-51 fluorescence microscope.

MTT proliferation assay

Cells (1×10^3) were seeded in 96-well plates in the presence of 200 μl of culture medium supplemented with 10% FBS. After 20–24 h, the cells were replaced in quintuplicate wells in the presence of 200 μl of serial dilutions of EGF–E4orf4 (2.5–250 nmol/l) or equimolar concentrations of EGF in media with 5% FBS. Following a 72-h incubation, the MTT colorimetric assay was performed according to the manufacturer's instruction using a microplate reader.

Cell colony-forming assay

Four hundred Saos-2 cells in 2 ml of DMEM containing 10% FBS were plated in six-well plates and incubated for 7 days in medium alone. On day 7, the medium was refreshed and the cells were assigned to four different treatments in quadruplicated wells: medium supplemented with 0.15 μmol/l EGF–E4orf4, 0.15 μmol/l, both EGF–E4orf4 and EGF in equal amounts, and fresh medium alone as control, respectively. Following 7 days of incubation, colonies were fixed, stained with crystal violet and scored. Results were expressed as percentage of colony formation [(mean no. of colonies/seeded cells) × 100] and as percentage inhibition of clonogenic cells using the formula: $1 - (\text{mean no. of colonies}_{\text{test}}/\text{mean no. of colonies}_{\text{control}}) \times 100$.

Flow cytometry

Cells were treated with 0.5 μmol/l of EGF–E4orf4 for 72 h. DNA fragmentation as one of the later steps in apoptosis was detected using the APO-BRDU kit (BD Biosciences San Diego, Califoria, USA) according to the manufacturer's manual. In the APO-BRDU assay, cells were fixed and incubated with BrdUTP in the presence of TdT enzyme in order to incorporate BrdUTP into the exposed 3′-OH termini of the double- and single-stranded DNA. After incorporation, these sites were identified by staining the cells with a FITC-labeled anti-BrdU mAb. Flow analysis was conducted using a BD Aria flow cytometer. Results from triplet independent experiments were statistically analyzed with SPSS 11.0 software.

Maltose-binding protein (MBP)–E4orf4 fusion protein and preparation of rabbit E4orf4 antibody

The E4orf4 coding sequence was generated by PCR using plasmid pUC-E4orf4 as template with sense primer 5′-ATG GTT CTT CCA GCT CTT CC-3′ and antisense primer 5′-GG GGA TCC TCA TTA CTG TAC GGA GTG CGC C-3′. The E4orf4 DNA fragment was cloned into the *Xmn*I–*Bam*HI sites of pMAL-c2X vector (New England Biolab, Boston, Massachusetts, USA). The recombinant plasmid was transformed into *Escherichia coli* BL21. The MBP–E4orf4 fusion proteins were produced by growing 500 ml of bacterial culture to an OD_{600} of 0.5; the culture was then induced with 0.3 mmol/l IPTG for 5 h. Cells were collected and sonicated in lysis buffer (50 mmol/l Tris, pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA and 1 mmol/l PMSF). The MBP–E4orf4 fusion proteins were purified using an amylose affinity column according to the manufacturer's instructions (New England Biolab). The purified MBP–E4orf4 fusion protein was injected into rabbits and then the immune sera passed through an amylose affinity column of immobilized MBP. Western blotting was applied to test the purified IgG fraction. The results showed that EGF–E4orf4 fusion protein was immunoreactive with purified IgG fraction and the size of the specific band was the same as that of the EGF–E4orf4 fusion protein reacting with the commercially available anti-hEGF antibody. The specific staining was only shown on the cells treated with EGF–E4orf4 by immunofluorescence analysis of the control cells versus the cells treated with EGF–E4orf4, the specific staining was only shown on the cells treated with EGF–E4orf4.

Plasmid construction, expression and purification of EGF–E4orf4

The generation of EGF–E4orf4 fusion protein has been described previously in Chinese [15]. Briefly, the α-factor-EGF and E4orf4 coding sequences were obtained by separate PCR using plasmid pUC-EGF and pUC-E4orf4 as template, respectively. The complementary sequence was introduced at the end of each fragment by primer design in order to enable the fragments to overlap each other during the annealing procedure. The fused fragment, containing the sequences encoding the α-factor signal peptide, hEGF, a short flexible linker and adenovirus E4orf4, was re-amplified with the forward primer of α-factor and the reverse primer of E4orf4. An 805-bp DNA fragment of EGF–E4orf4 was cloned into the yeast expression vector pAO815 (Invitrogen, Life Technologies, Carlsbad, California, USA). The plasmid pAO-3EGF-E4orf4 containing three expression cassette copies was thus constructed from pAO-EGF-E4orf4 according to the instructions provided by Invitrogen. *P. pastoris* strain GS115 was transformed with plasmid pAO-3EGF-E4orf4 for the expression of the fusion protein. EGF–E4orf4 fusion proteins were purified by ion-exchange chromatography. SDS–PAGE showed a specific protein band with an apparent molecular weight of 20 kDa, which correlated well with the predicted molecular mass of 19973.4 Da. Immunoblot analysis showed that the full-length fusion proteins were immunoreactive against antibodies to EGF and E4orf4.

Results

Binding and internalization of EGF–E4orf4 by Saos-2 cells

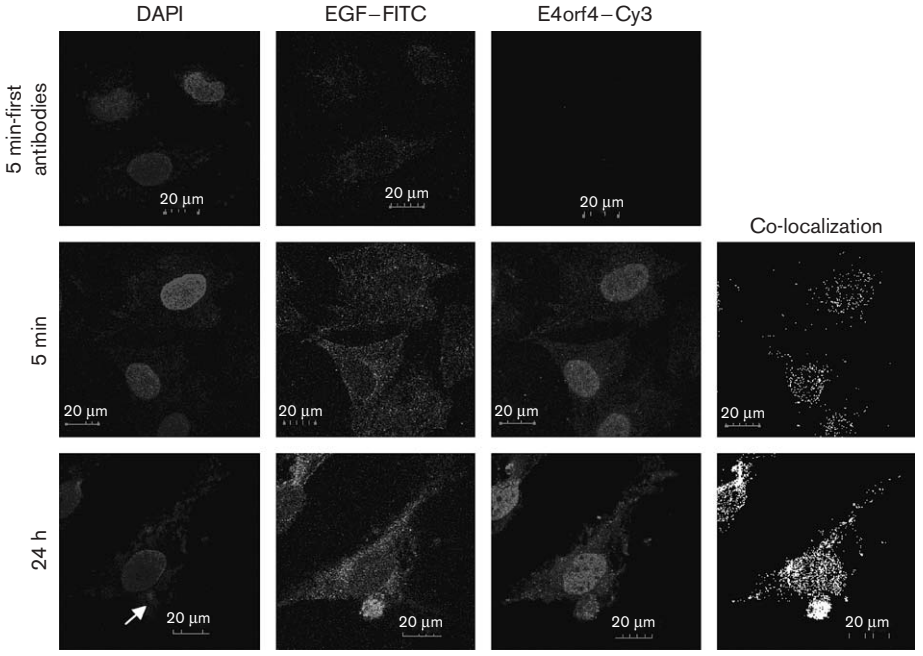
In order to examine whether EGF–E4orf4 was binding to the cell surface and then taken up by Saos-2 cells, immunocytochemistry and confocal laser microscopy were performed. The results demonstrated that Saos-2 cells easily internalized EGF–E4orf4 (Fig. 1). As early as 5 min after exposure to EGF–E4orf4, EGF and E4orf4 co-localization on the cell membrane, cytoplasm and even in the nucleus was detected as green fluorescence against anti-EGF antibody and as red fluorescence against anti-E4orf4 antiserum (Fig. 1, middle row). To examine the kinetics of EGF–E4orf4 uptake by the cells, we traced EGF and E4orf4 for 24 h after addition to the culture medium. The fluorescence signals of EGF and E4orf4 were more brightly shown in the cytoplasm and nuclei (Fig. 1, bottom row). Co-localization of green–red fluorescence emission signals was quantitatively assessed with FluoView software and displayed as in Fig. 1 (right). It was clearly shown that the specimen taken from 24 h (bottom right) had more co-localization between the two fluorophores than the specimen from 5 min (Fig. 1, middle right) and most of the overlap plots were condensed in the cell nucleus.

We next examined EGFR/EGF–E4orf4 using anti-EGFR antibody and anti-E4orf4 antiserum in Saos-2 cells treated with EGF–E4orf4 for 5 min and 24 h (Fig. 2). As expected, the EGFR expression was shown in the Saos-2 cells (Fig. 2a, green fluorescence, middle left). The EGFR signals were mainly bound to the cell surface at 0 and 5 min, and decreased at 24 h. EGF–E4orf4 not only remained in the cytoplasm, but mostly in the nuclei, stained as red fluorescence (Fig. 2a, middle right), whereas only a few of the co-localized EGFR and EGF–E4orf4 signals were found in FluoView software analysis from 5 min (Fig. 2b). After a 24-h exposure to EGF–E4orf4, some of the cells showed morphological changes consistent with apoptosis, including marked shrinkage, irregular shape or even disintegration (Fig. 1 bottom left and Fig. 2a, bottom left, arrows).

Inhibition of proliferation of Saos-2 cells by EGF–E4orf4

MTT proliferation assay was performed to determine if the EGF–E4orf4 fusion protein could inhibit tumor cell growth. Saos-2 cells, which express EGFR, were used to evaluate the cytotoxicity of the EGF–E4orf4 fusion protein. Cells were cultured in medium in the presence of various concentrations of EGF–E4orf4 and in medium alone as control. This fusion protein exhibited a

Fig. 1



Co-localization of EGF and E4orf4 in Saos-2 cells. Saos-2 cells were incubated with EGF–E4orf4 for 5 min (middle row) and 24 h (bottom row). Double immunostaining using anti-hEGF (green fluorescence, middle left) and anti-E4orf4 (red fluorescence, middle right) was performed as described in Materials and methods. Blue fluorescence (left) denotes the nuclei stained with DAPI. Specimens were analyzed by fluorescence confocal microscopy. Co-localization of green–red fluorescence emission signals was quantitatively assessed with FluoView software and shown as white spots (right). In the top row, Saos-2 cells were treated with EGF–E4orf4 for 5 min and we performed the same immunodetection procedures except incubated with the specific antibodies against EGF and E4orf4; no green and red fluorescent staining was observed. Arrow indicates the disintegrated nucleus (bottom row).

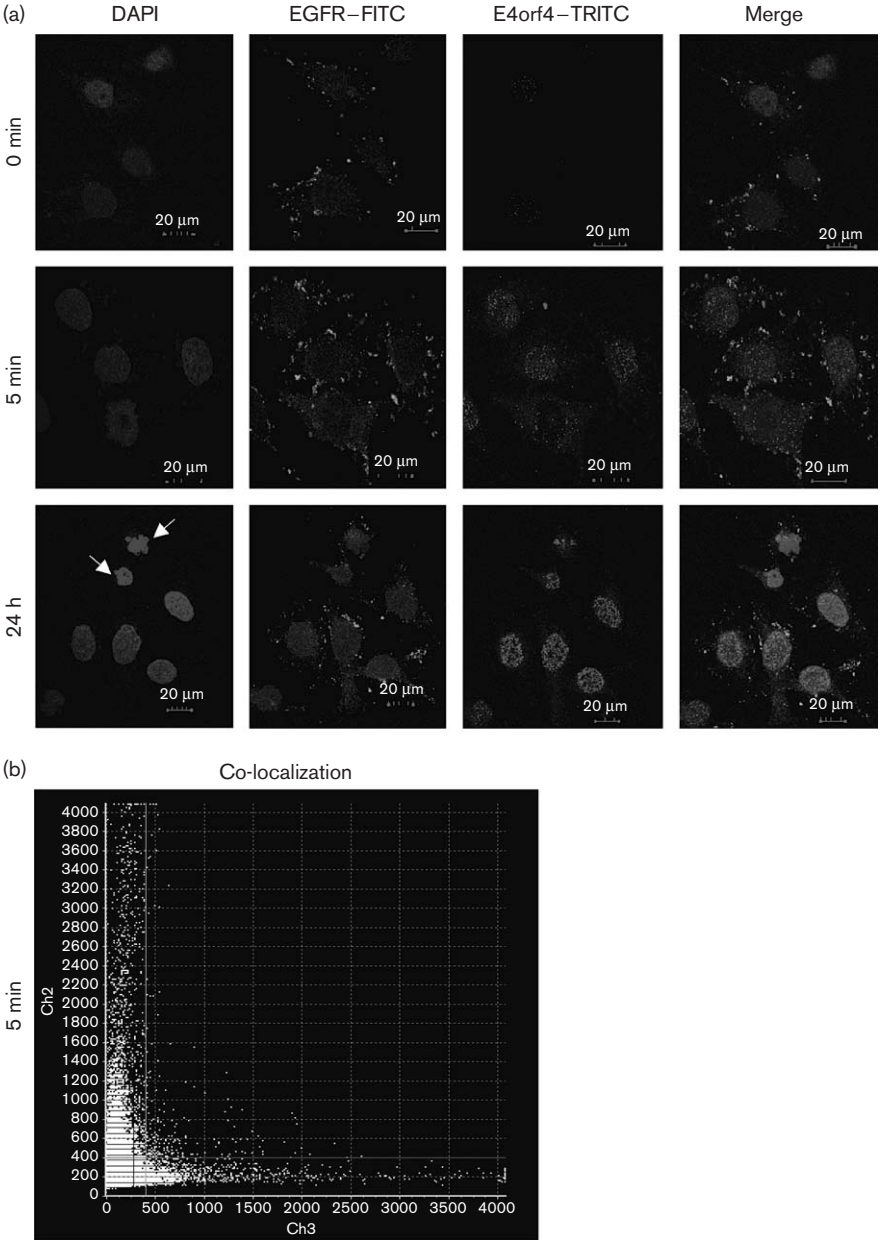
dose-dependent cytotoxic reaction. There was a slight proliferation of the EGF–E4orf4-treated Saos-2 cells at low concentrations of EGF–E4orf4 as compared with the control cells. When concentrations of EGF–E4orf4 were increased up to the range of 62.5–250 nmol/l, the growth of Saos-2 cells gradually diminished until nearly 50% of the cells were killed (Fig. 3a). In contrast, cells treated with equimolar concentrations of EGF only showed increasing proliferation (Fig. 3a). Three independent experiments showed the same results.

To test whether E4orf4 kills transformed cells specifically, HUVEC cells, which express EGFR [36], were incubated with EGF or EGF–E4orf4 fusion protein. Figure 3(b) shows that the cell growth curves were almost the same in both treatments. In other words, EGF–E4orf4 exerted no significant inhibitory effect on the primary cells even at a concentration of EGF–E4orf4 as high as 250 nmol/l.

Cytotoxicity of EGF–E4orf4 on colony growth of Saos-2 cells

Saos-2 cells were plated and incubated overnight. The medium was then aspirated and replaced by fresh medium or medium containing EGF–E4orf4. Following 7 days of incubation, colony formation of cells appeared in the control medium, but not in the EGF–E4orf4-treated cells. We tried 3 times using different numbers of seeded cells and different concentrations of EGF–E4orf4. Unfortunately, to no avail because EGF–E4orf4 completely nullified the ability of Saos-2 cells to form colonies. Subsequently, the inhibitory capability of EGF–E4orf4 on the pre-established colonies was determined as described in Materials and methods. The results shown in Fig. 4 indicated that EGF–E4orf4 reduced colony growth as compared to the cells cultured in plain medium. The *P* values for the one-way ANOVA test comparison of the mean clonogenic cell survival for EGF–E4orf4 versus the cells cultured in the plain medium was significant at the 0.05 level. The percentages of inhibition of clonogenic cells treated with EGF–E4orf4 from two independent experiments were 31.0 and 41.4%. In contrast, EGF did not affect the clonogenic growth of Saos-2 cells. Cells incubated with EGF–E4orf4 plus equimolar concentrations of EGF showed a slight increase in the number of colonies compared with cells treated with EGF–E4orf4. This means that the inhibition activity in the established colonies was partially blocked by competition from the same concentration of EGF.

Fig. 2

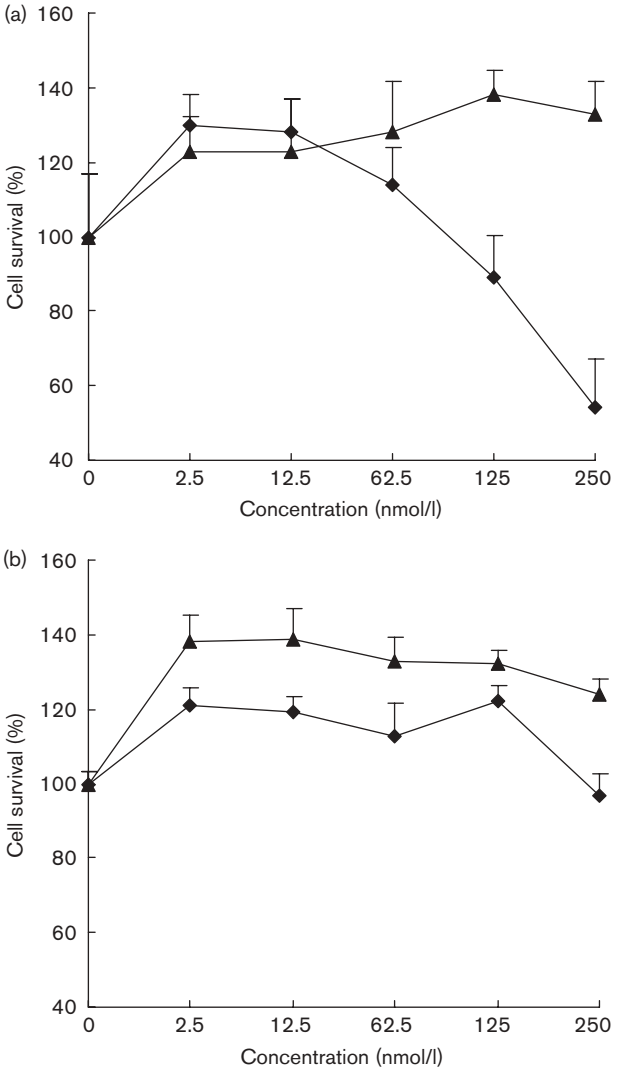


Immunodetection of EGFR and EGF–E4orf4 in Saos-2 cells. Cells were incubated with EGF–E4orf4 for the indicated times (a, 0 min, top; 5 min, middle; 24 h, bottom) and processed for the detection of EGFR (green fluorescence, middle left) and EGF–E4orf4 (red fluorescence, middle right) by immunocytochemistry. Blue fluorescence denotes the nuclei stained with DAPI (left). A merged image of FITC, TRITC and DAPI fluorescence (right) is visualized. In (a), the EGFR signal was mainly detected on the cell surface of Saos-2 cells either untreated (top) or treated with EGF–E4orf4 for 5 min and 24 h (middle and bottom). In the bottom row, by 24 h, some of the cells showed morphological changes consistent with apoptosis (arrows). In (b), the co-localization between the two green and red fluorophores was analyzed with FluoView software in the slide taken from 5 min and only a few white spots were located in the coordinate district.

EGF–E4orf4 induced apoptosis in Saos-2 cells

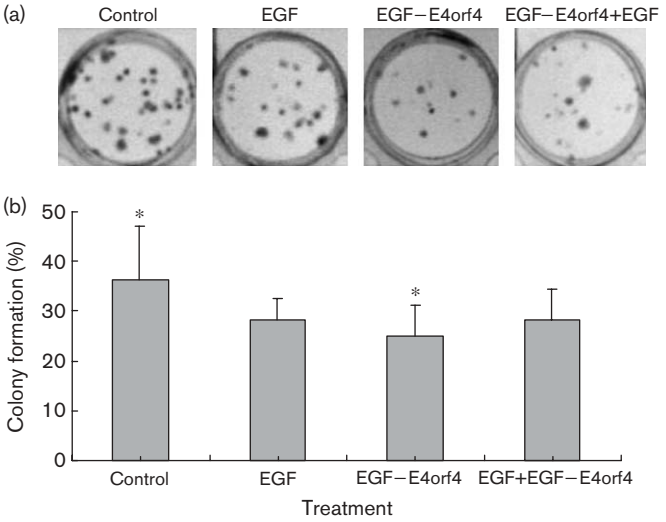
Compared with cells incubated with medium alone, part of the Saos-2 cells treated for 72 h with 0.3 μmol/l EGF–E4orf4 became round. Some were disrupted and detached from the surface of the cell culture dishes as viewed under the reverse light microscope. For testing induced apoptosis of EGF–E4orf4, Saos-2 cells were stained with DAPI and subjected to fluorescence microscopy. The number of EGF–E4orf4-treated cells was much less than the number of control cells (Fig. 5, left). The nuclei of the cells incubated with medium alone were weakly and uniformly stained with DAPI,

Fig. 3



EGF–E4orf4 fusion protein inhibits the growth of Saos-2 cells, but not the primary HUVECs. Saos-2 cells (a) and HUVECs (b) were incubated with the indicated concentrations of EGF–E4orf4 (♦) or EGF (▲) for 72 h, and cytotoxicity was determined by MTT assay. The values shown are means ± SD from five replicated wells for each treatment. Results are expressed as percentage of the OD value of the control cells incubated in medium alone.

Fig. 4



EGF–E4orf4 fusion protein reduces colony formation of Saos-2 cells. Four hundred Saos-2 cells were seeded into each well. Following a 7-day incubation, cell colonies were treated with either the fresh medium alone, EGF, EGF–E4orf4 or EGF plus EGF–E4orf4, as indicated, for an additional 7 days. Colonies were fixed and stained with Crystal violet. The values shown are means ± SD from four replicated wells for each treatment. Percentages of surviving cells were obtained by counting the number of resulting colonies and are expressed relative to the total number of seeded cells.

indicating that they were intact (Fig. 5, top right), whereas nuclei from cells treated with EGF–E4orf4 shrunk or showed irregularity in shape, characterized by intense staining of highly condensed chromatin (Fig. 5, bottom right).

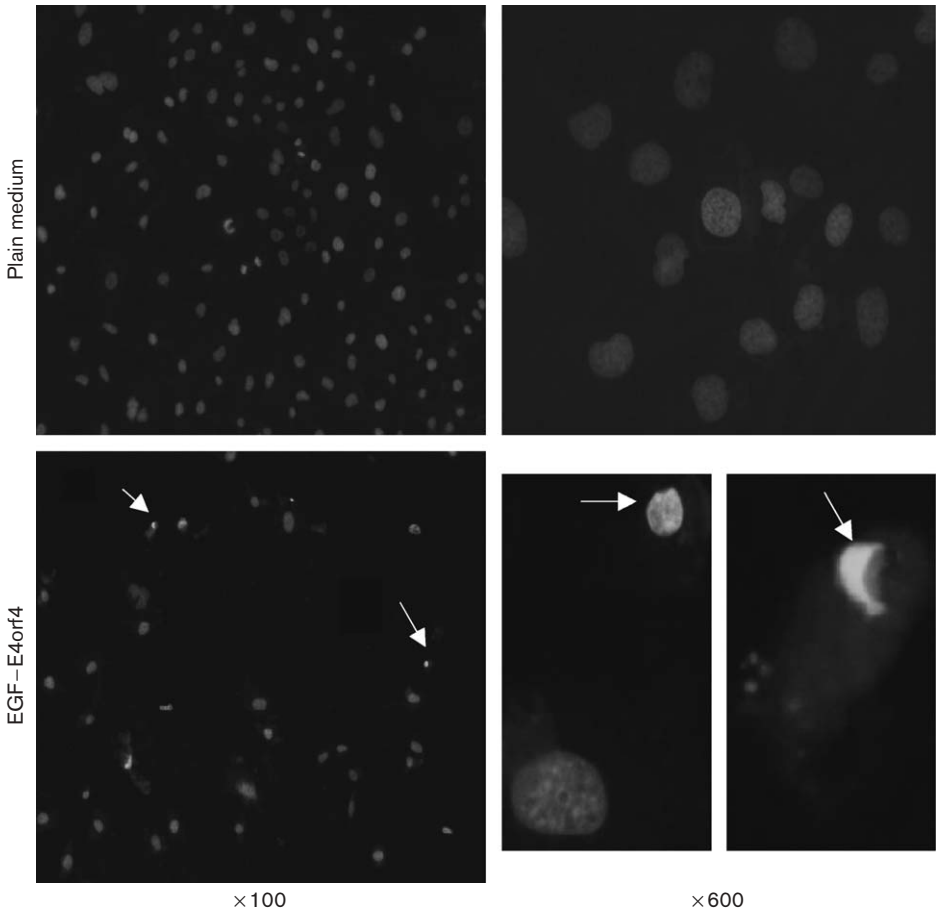
To further confirm the ability of EGF–E4orf4 to induce apoptosis, cells were fixed and DNA fragmentation was detected using the APO-BRDU kit. Flow cytometric analysis showed the predicted results on control cells provided by the APO-BRDU kit in which non-apoptotic cells did not incorporate significant amounts of BrdUTP due to the lack of exposed 3′-OH ends and consequently have relatively little fluorescence compared to the apoptotic cells that have an abundance of 3′-OH ends (Fig. 6a). When Saos-2 cells were treated with 0.5 μmol/l EGF–E4orf4 for 72 h, EGF–E4orf4-induced apoptosis was shown by an increase in the number of cells staining with anti-BrdU-FITC mAb (Fig. 6b). The results from three independent experiments revealed significant increases of 38.6, 27.3 and 21.9%, respectively, of apoptotic cells in the Saos-2 cells treated with EGF–E4orf4, as compared with cells cultured in plain medium ($t = 0.028$, $P < 0.05$).

Discussion

We employed recombinant DNA methodologies for gene construction in which hEGF and adenovirus E4orf4 were joined by a flexible short linker [15]. The fusion protein EGF–E4orf4 presented combined characteristics of the cytotoxicity of adenovirus E4orf4 and the cell-targeting specificity of EGF. In the current study, we present experimental evidence that the recombinant fusion protein EGF–E4orf4 was capable of entering cells mediated by the EGFR and thus inhibited the growth of human tumor cells, i.e. Saos-2 cells, but not the primary HUVECs. The data also indicated that EGF–E4orf4 induced apoptosis in Saos-2 cells.

Drug-targeted therapy is a new, developing anti-cancer treatment enabling drugs to be carried to tumor cells,

Fig. 5

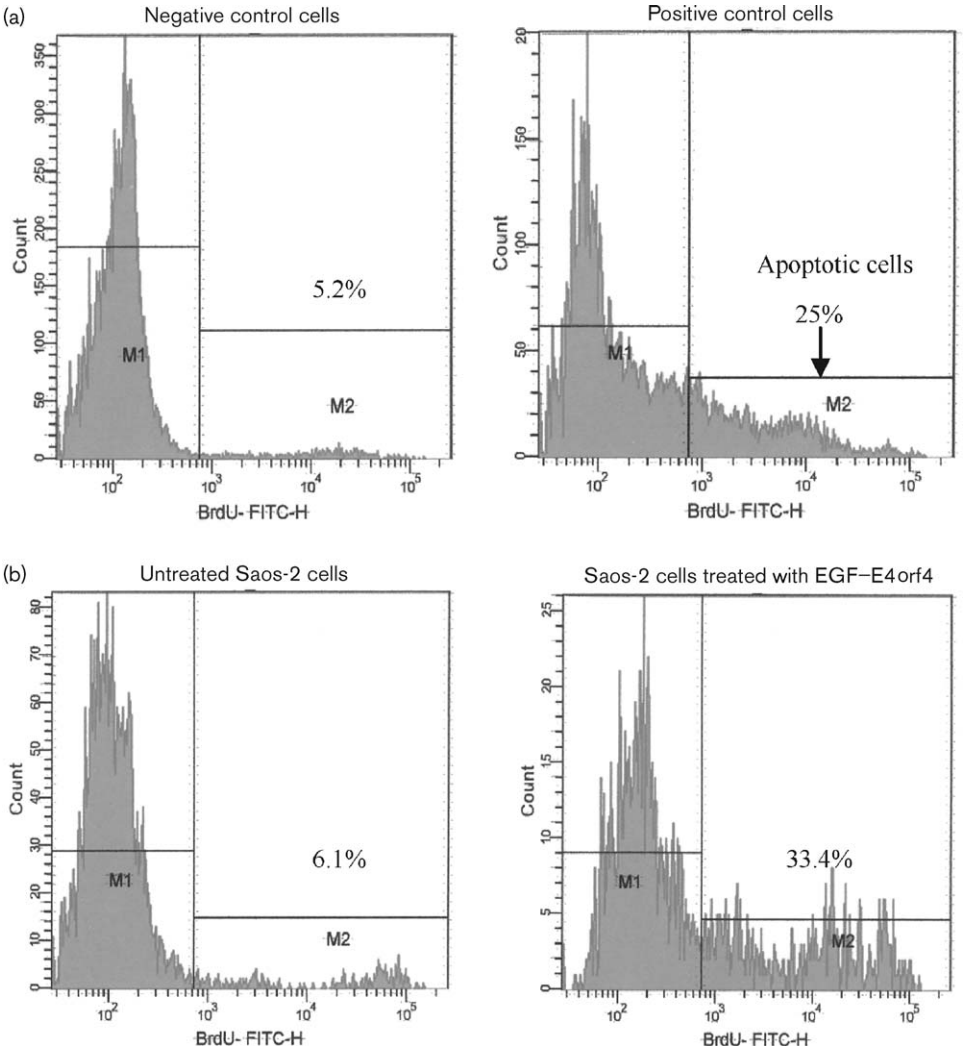


EGF–E4orf4 fusion protein induces apoptosis in Saos-2 cells (morphological identification). Cells were incubated with medium alone (top row) or 0.3 μmol/l EGF–E4orf4 (bottom row) for 72 h. The cells were fixed and stained with DAPI, and subjected to fluorescence microscopy at magnifications of × 100 (left) and × 600 (right).

either by killing the cells directly or via a specific effective pathway, thus producing a higher therapeutic index and less toxicity than conventional therapies. EGFR is highly expressed in a variety of human solid tumors, including cancers of the prostate, breast, lung, stomach, colorectum, ovary, etc. [16,17]. Activation of EGFR has been shown to play a particularly prominent role in the processes of tumor cell growth such as vascularization, invasiveness and metastasis. Therefore, high expression of EGFR is correlated with a poor response to treatment, disease progression and poor survival [14]. Thus, such crucial importance of EGFR to carcinomas infers that EGFR is an attractive target for cancer therapy. Over the past two decades, much effort has been directed towards developing anti-cancer agents that can interfere with EGFR activity. Contemporary approaches in development for targeting the EGFR include: anti-erbB/EGFR monoclonal antibodies; small-molecule inhibitors of EGFR tyrosine kinase enzymatic activity; recombinant proteins containing EGF or transforming growth factor-α fused to toxins; EGFR-directed vaccines; EGFR-mediated gene therapy; and antisense oligonucleotides specific for EGFR mRNA [17–21].

In the present study, we have employed the approach of an EGFR ligand–toxin in EGFR-positive Saos-2 tumor cells. This approach has been reported previously in which the ligand–toxin contained an EGFR-binding moiety (either an antibody fragment or an EGFR ligand) conjugated to a potent cellular toxin such as genistein (a component of soy) [22,23], diphtheria toxin [24,25], *Pseudomonas aeruginosa* toxin [26–28], angiogenin [29] or eosinophil cationic protein [30]. Although these compounds have demonstrated anti-cancer activity in xenograft tumor models and cell culture, most have yet not entered clinical testing. Thus, the new agents still merit further investigation.

Fig. 6



EGF–E4orf4 induces apoptosis in Saos-2 cells (flow cytometric analysis). (a) The control cells were supplied by the APO-BRDU kit. Non-apoptotic cells (M1 gates) do not incorporate significant amounts of BrdUTP due to the lack of exposed 3′-OH ends and consequently have relatively little fluorescence compared to apoptotic cells which have an abundance of 3′-OH ends (M2 gates). (b) Saos-2 cells were untreated or treated with 0.5 μmol/l EGF–E4orf4 for 72 h. Cells were fixed and apoptotic cells were detected using the APO-BRDU kit.

The newly confirmed death factor adenovirus E4orf4 was used in our study. Our results from immunofluorescence assays combined with confocal laser microscopy demonstrated that the EGFRs were overexpressed on the cell surface of the human osteosarcoma cell line Saos-2 (Fig. 2). EGF–E4orf4 fusion protein enabled binding onto the cell surface and then was internalized into the Saos-2 cells. Within 5 min after the cells were exposed to EGF–E4orf4, the latter began to be internalized, as determined by co-localization of EGF and E4orf4 on the cell membrane, in the cytoplasm and even in the nucleus (Fig. 1, middle row). This means that the receptor-binding affinity of EGF–E4orf4 was not affected despite its much larger molecular weight than EGF. After a 24-h incubation of Saos-2 cells with EGF–E4orf4, the co-localization signals of EGF and E4orf4 were increased, and most of them were condensed in the cell nuclei (Fig. 1, right). Meanwhile, some of the cells showed morphological changes consistent with apoptosis, including marked shrinkage, irregular shape or even disintegration (Fig. 1, bottom left and Fig. 2a, bottom left, arrows). E4orf4 is a small 114-residue protein and contains an arginine-rich motif (ARM)-like sequence between residues 66 and 75 (E4ARM). E4ARM is highly homologous to the ARM of other lentiviral proteins such as those of HIV-1 Tat [31], HIV-1 Rev [32] and HTLV-1 Rex [33]. Branton and Roopchand [4] have reported that high quantities of E4orf4 were found in cell nuclei following overexpression. The study from Miron *et al.* [34] conferred the functions of E4orf4 in the nucleus. They

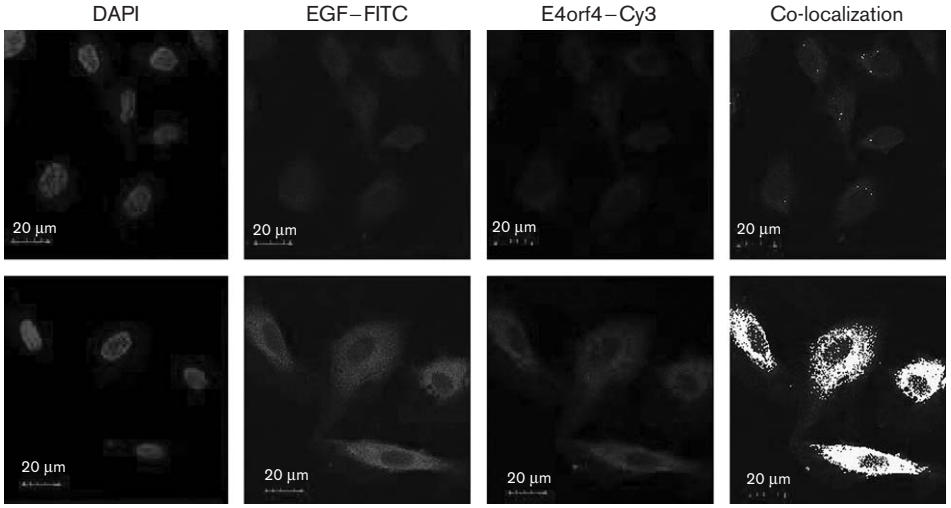
demonstrated that the 10 residues of E4ARM were necessary and sufficient to target E4orf4 to the nucleus and nucleolus as well as to regulate its ability to induce cell death. The fact that the fusion protein EGF–E4orf4 was found to localize in the nuclei within only 5 min after being delivered into the cells and was mostly retained in the nuclei for as long as 24 h provides further evidence to indicate the important roles of the nuclear localization of E4orf4. The role of E4orf4 in the nucleus is not yet clearly understood. Further investigations will be conducted in order to identify the E4orf4-associating proteins in the nucleus.

Assessment of the biological function of EGF–E4orf4 was monitored with p53-deficient human Saos-2 cells. The MTT colorimetric assay revealed that EGF–E4orf4 slightly promoted proliferation of Saos-2 cells at low concentrations (2.5–12.5 nmol/l), which exhibited a similar situation as for EGF. As the concentration was raised to 125 nmol/l, the cytotoxic effect of EGF–E4orf4 became strikingly prominent and nearly 50% of the Saos-2 cells were killed when the concentration was increased to 250 nmol/l. Nevertheless, only the proliferation effect on Saos-2 cells was demonstrated with EGF treatment regardless of concentrations varying from 2.5 to 250 nmol/l (Fig. 3a). We presume that the fusion protein EGF–E4orf4, first, specifically binds to EGFR via EGF on the cell membrane and then is internalized by endocytosis. As mentioned above, at low concentrations of EGF–E4orf4, a similar effect on Saos-2 cell proliferation as for EGF was observed, because EGF is the first to function and its effect concealed the yet weak inhibitory effect of E4orf4. With increasing intracellular concentration of EGF–E4orf4, the role of E4orf4 gradually increases to inhibit the growth of Saos-2 cell by distinct mechanisms. Similar dose-dependent cytotoxicity has also been revealed in EGFR-positive MDA-MB-231 (human breast cancer) and BT325 (human glioblastoma) cell lines, but with variant IC_{50} dosage values [15]. In this study we have not further tested the sensitivity of different cell lines to EGF–E4orf4. The observation among three cell lines with different IC_{50} dosage values of EGF–E4orf4 may depend on the uptake of EGF–E4orf4 into the cell, which is correlated with the number of EGFRs expressed on the cell surface. The positive correlation between cytotoxicity of EGF–toxin conjugate and EGFR expression level has already been described by Shaw *et al.* [24] in that some tumor cells, which express large numbers of EGFRs, were sensitive to $DAB_{389}EGF$, whereas some other tumor and normal tissue cell lines expressing few or no EGFRs were markedly insensitive. Jinno *et al.* [30] obtained the same results on their study of EGF–ECP cytotoxicity in human breast cancer cell lines. Their results inferred that the IC_{50} values of the conjugate decreased along with the increasing numbers of EGFRs of the cancer cell line.

EGF–E4orf4 treatment of Saos-2 cells induced apoptosis that was evidenced by the appearance of condensed and irregular-shaped nuclei after DAPI staining (Fig. 1, bottom left, Fig. 2a, bottom left, arrows and Fig. 5, bottom row). FACS detection using the APO-BRDU kit revealed a significant population increase of the EGF–E4orf4-treated Saos-2 cells stained with anti-BrdU-FITC mAb, which was indicative of apoptotic death (Fig. 6b). The results were in concert with those of Marcellus *et al.* [5] on E4orf4-induced, p53-independent apoptosis in Saos-2 cells, although in our study E4orf4 was internalized into the cells as a protein reagent. Moreover, in previous studies our group had detected changes in the cell cycle of EGF–E4orf4-treated Saos-2 cells and observed no increase in the number of sub-G_1 cells when Saos-2 cells were incubated with a low concentration of EGF–E4orf4 (0.15 μmol/l). However, cell cycle effects were detected in which there was a significant decrease (18.0%) in the percentage of cells in the G_2 phase as compared with those incubated in medium alone (27.9%). It was estimated that 56.3% of the EGF–E4orf4-treated cells arrested in the G_1 phase, while only 49.5% of G_1 phase cells were found in the cells incubated with medium alone (data not shown). In addition, the induction of cell cycle arrest by the fusion protein EGF–E4orf4 was not an occasional phenomenon that happened on Saos-2 cells, but was also detected in MDA-MB-231 cells and A549 cells (data not shown). This finding was in agreement with the results from Kornitzer *et al.* [7], who reported that in a stably transfected 293-derived cell line, expressing E4orf4 from a tetracycline-regulated promoter, G_2/M arrest was observed after a 24-h induction with doxycycline. After a 48-h induction, the G_2/M arrest was released and cells with sub-G_1 DNA content undergoing apoptosis started to accumulate. These results indicate that, under certain physiological conditions, E4orf4 can initially induce cell cycle arrest, from which the cells eventually escape and undergo apoptosis. However, the results of cell cycle analysis presented in our study did not agree with those reported from other studies of adenovirus E4orf4, which had been found to cause G_2/M arrest both in mammalian 293 cells [7] and in yeast cells [7,8]. Furthermore, a recent study on the mechanism of cell cycle effects induced by viral proteins has shown that E4orf4 induces G_2/M arrest by means of targeting and inhibiting the anaphase-promoting complex/cyclosome [35]. Whether the G_1 arrest in Saos-2 cells given in our study designated a different mechanism of cell killing by E4orf4 is not clear. Based on these rather preliminary results, further experiments are warranted in order to understand the pathway or mechanisms on E4orf4-induced apoptosis and cell cycle arrest after the entry of E4orf4 into the cells as a protein reagent.

The work presented here also demonstrated the selectivity of EGF–E4orf4 in killing cancer cells only. Using primary cells, i.e. HUVECs, which express EGFR [36],

Fig. 7



Co-localization of EGF and E4orf4 in HUVECs. Cells were incubated with EGF–E4orf4 for 30 min, and double immunostaining using anti-hEGF (green fluorescence, middle left) and anti-E4orf4 (red fluorescence, middle right) was performed as described in Materials and methods. Blue fluorescence (left) denotes the nuclei stained with DAPI. Specimens were analyzed by fluorescence confocal microscopy. Co-localization of green–red fluorescence emission signals was quantitatively assessed with FluoView software and shown as white spots (right). In the top row, cells were treated with EGF–E4orf4 for 30 min, and we performed the same immunodetection procedures except incubated with the specific antibodies against EGF and E4orf4; no green and red fluorescent staining was observed.

EGF–E4orf4 exhibited the same proliferation effect as EGF and only little effect when the concentration of EGF–E4orf4 was raised to 250 nmol/l (Fig. 3b). Furthermore, we performed immunocytochemical tests combined with confocal laser microscopy to observe whether EGF–E4orf4 also enter the HUVECs. When using anti-EGFR antibody in HUVECs, the signals of EGFR were not like the signals in Saos-2 cells, which were clearly shown by the spots around the cell surface. The signals were weaker and spread all over the cells in HUVECs (data not shown). This may suggest that HUVECs express EGFR, but with at lower level than Saos-2 cells. We detected EGF–E4orf4 using anti-EGF antibody and anti-E4orf4 antiserum in HUVECs treated with EGF–E4orf4 for 30 min. The results confirmed that EGF–E4orf4 was able to enter the HUVECs, and fluorescence signals of EGF and E4orf4 mainly co-localized in the cytoplasm (Fig. 7). The result of less toxicity to HUVECs is consistent with the previous reports, although E4orf4 is, as a protein reagent, transferred into the cells via EGFR. Using transformed and primary normal rat cells, Shtrichman *et al.* [3] found that E4orf4 kills oncogene-expressing cells much more efficiently than untransformed cells. E4orf4 had little effect on about a dozen primary human cell types derived from various tissues as reported by Branton and Roopchand [4]. E4orf4, which induces p53-independent apoptosis and kills transformed cells preferentially, is thus of significant cancer therapeutic interest [37]. It is unclear yet why E4orf4 exhibited specificity to cancer cells. In view of the fact that E4orf4 mainly located in the cell nucleus of Saos-2 cells and remained in the cytoplasm of HUVECs, however, such a difference in E4orf4 localization gives us a clue for further investigation. We will examine other primary cells to test the selective cytotoxicity of EGF–E4orf4. Although the mechanism of E4orf4's specificity in killing cancer cells awaits resolution, it is worth emphasizing, with respect to cancer therapy, the crucial importance of its ability in exerting the least adverse effect to normal cells while implying more efficiency in targeting against tumor cells.

Our preliminary data show that recombinant fusion protein EGF–E4orf4 has a potent effect on growth inhibition of p53-deficient tumor cells and it seems promising for further development of relevant reagents, hopefully for the treatment of p53-null human cancers.

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