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Research article

CORRELATION BETWEEN THE LEVELS OF SURVIVIN AND SURVIVIN PROMOTER-DRIVEN GENE EXPRESSION IN CANCER AND NON-CANCER CELLS

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Abstract: Survivin, a member of the inhibitor of apoptosis (IAP) protein family, is associated with malignant transformation and is over-expressed in most human tumors. Using lipoplex-mediated transfection, we evaluated the activity of the reporter enzyme, luciferase, expressed from plasmids encoding the enzyme under the control of either the cytomegalovirus (CMV) or survivin promoters, in tumor- and non-tumor-derived human and murine cells. We also examined whether there is a correlation between the survivin promoter-driven expression of luciferase and the level of endogenous survivin. Human cancer cells (HeLa, KB, HSC-3, H357, H376, H413), oral keratinocytes, GMSM-K, and chemically immortalized human mammary cells, 184A-1, were transfected with Metafectene at 2 µl/1 µg DNA. Murine squamous cell carcinoma cells, SCCVII, mouse embryonic fibroblasts, NIH-3T3, and murine immortalized mammary cells, NMuMG, were transfected with Metafectene PRO at 2 µl/1 µg DNA. The expression of luciferase was driven by the CMV promoter (pCMV.Luc), the human survivin promoter (pSRVN.Luc-1430), or the murine survivin promoters (pSRVN.Luc-1342 and pSRVN.Luc-194). Luciferase activity was measured, using the Luciferase Assay System and expressed as relative light units (RLU) per ml of cell lysate or per mg of protein. The level of survivin in the lysates of human cells was determined by ELISA and expressed as ng survivin/mg protein. In all cell lines, significantly higher luciferase activity was driven by the CMV promoter than by survivin promoters. The expression of

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Abbreviations used: DMEM - Dulbecco's modified Eagle's MEM medium; FBS - fetal bovine serum; HNSCC - head and neck squamous cell carcinoma; IAP - inhibitor of apoptosis; OSCC - oral squamous cell carcinoma; RLU - relative light units

luciferase driven by the CMV and survivin promoters in murine cells was much higher than that in human cells. The cells displayed very different susceptibilities to transfection; nevertheless, high CMV-driven luciferase activity appeared to correlate with high survivin-promoter driven luciferase expression. The survivin concentration in lysates of cancer cells ranged from 5.8 ± 2.3 to 24.3 ± 2.9 ng/mg protein (mean, 13.7 ng/mg). Surprisingly, elevated survivin protein was determined in lysates of non-tumor-derived cells. Survivin levels for GMSM-K and 184A-1 cells, were 16.7 ± 8.7 and 13.5 ± 6.2 ng/mg protein, respectively. The expression of endogenous survivin did not correlate with the level of survivin promoter-driven transgene activity in the same cells. The expression of survivin by non-tumorigenic, transformed cell lines may be necessary for their proliferative activity. The level of survivin promoter-driven gene expression achieved via liposomal vectors in OSCC cells was too low to be useful in cancer-cell specific gene therapy.

Key words: Transfection, Survivin, Metafectene, Metafectene PRO, Survivin promoter, Non-cancer cells, CMV promoter, Oral squamous cell carcinoma cells

INTRODUCTION

Carcinogenesis is a multi-factorial process involving the activation of oncogenes and the inactivation of tumor suppressor genes. Most human tumors are characterized by an imbalance of regulatory mechanisms controlling the cell cycle, the cell death/cell viability balance, and apoptosis [1, 2]. Apoptosis has become an important tool in developing new cancer strategies. In addition to pro- and anti-apoptotic Bcl-2 molecules, a second family of inhibitors of apoptosis (IAP) has been identified recently [3]. Survivin, a 16.5 kDa protein also known as baculoviral IAP repeat-containing protein 5 (BIRC5), represents a bi-functional IAP involved in the regulation of cell division at the G_2/M phase and in the inhibition of apoptosis. Expressed at mitosis in a cell cycle-dependent manner and physically associated with the mitotic apparatus, survivin is essential for completion of various stages of cell division via regulation of microtubule dynamics and stability [4-7]. Survivin suppresses apoptosis by interfering with caspase-9 processing, the upstream initiation of the intrinsic (mitochondrial) pathway of apoptosis [8].

Unlike other members of the Bcl-2 and IAP families, survivin has a unique expression profile. It is strongly upregulated in embryonic and fetal organs**,** but is essentially undetectable in most terminally differentiated normal tissues [9, 10], with the exception of thymocytes, CD34+ bone marrow-derived stem cells, and intestinal basal crypt epithelial cells [11]. A high re-expression of survivin has been shown in human tumors of lung, breast, colon, stomach, esophagus, pancreas, liver, uterus, ovaries, large cell non-Hodgkin's lymphoma, leukemias, neuroblastoma, pheochromocytoma, soft-tissue sarcomas, gliomas, melanoma, and non-melanoma skin cancers [12, 13].

Survivin is expressed in approx. 60-90% of oral squamous cell carcinomas (OSCCs) [14-18]. The cancer-specific expression of survivin makes it (i) a useful diagnostic marker of oral cancer [14] and (ii) a potential target for cancer treatment such as gene therapy [18-21]. The expression of survivin is an early event during oral carcinogenesis and may be useful for the identification of pre-cancerous lesions at higher risk of progression into invasive carcinoma [14, 15, 17]. Most OSCCs develop in the presence of clinical pre-malignant lesions [22]. Erythroplasias and dysplastic leukoplakias are the most common potentially malignant lesions, and about half of OSCCs exhibit associated leukoplakia [23]. Worldwide, OSCC is the most frequent malignant tumor of the oral cavity, and the sixth most common cancer in humans [24]. The high expression of survivin is associated with the more aggressive and invasive phenotype of OSCC [18, 25, 26]. Fifty two per cent of positively diagnosed patients have a mean survival time of only five years [27], a statistic that has not changed appreciably over the past 20 years. Even when the best combination of surgical and non-surgical treatments is used, more than 50% patients with OSCC will be affected by relapse, either locally, in regional lymph nodes, or at a distant site [24, 28].

Gene therapy, which represents a new approach to the treatment of cancer, is based on the hypothesis that specific genes can be introduced into tumor cells to mediate a direct or indirect anti-tumor effect. Recently, several pre-clinical studies have shown promising results of gene therapy for the treatment of OSCC. Due to its frequent genetic mutations and accessibility for intra-tumor injection, OSCC is an especially appropriate target for gene therapy [29-31]. Most gene therapy approaches use non-specific strong prokaryotic promoters (CMV and SV40) that can be expressed at high levels in normal cells, potentially contributing to toxicity. An alternative approach would be to utilize tissue- or tumor-specific promoters to restrict the therapeutic gene expression to cancer cells [32, 33]. Since the survivin gene has essentially no transcriptional expression in normal tissues, the survivin promoter could be employed to enhance the specificity of therapeutic gene expression in OSCC cells.

Here, using lipoplex-mediated transfection we evaluated the survivin- and the CMV promoter-driven expression of luciferase in human OSCC cell lines and SCCVII murine squamous carcinoma cells. The main purpose of our study was to compare (in the same cells) survivin expression driven by chromosomal survivin promoters (endogenous survivin) with survivin expression from plasmids driven by recombinant survivin promoter (exogeneous survivin) delivered by non-viral vectors. Non-tumor derived human and murine cells were also included in the study. To assess whether the survivin promoter-driven luciferase expression is correlated to the level of survivin, we measured survivin using ELISA in the lysates of cells that were used in transfection experiments. To our knowledge, this is the first study that compares the survivin promoter-driven expression of a reporter gene with the survivin protein levels measured by a quantitative assay. The murine cell lines were included in our study because

we are interested in establishing an efficient, tumor-specific non-viral gene delivery system for the treatment of OSCCs in immunocompetent C3H/HeJ mice. This orthotopic murine model for OSCC involves the injection of SCCVII murine squamous cell carcinoma cells in the floor of the mouth [34, 35]. Some of our results have been presented earlier in preliminary form [36, 37].

MATERIALS AND METHODS

Materials

Metafectene^{TM} and Metafectene PRO^{TM}, polycationic liposomal transfection reagents, containing a polyamine-lipid and dioleoyl phosphatidylethanolamine (DOPE), were purchased from Biontex Laboratories GmbH (Munich, Germany). This new class of transfection reagents has been developed based on the Repulsive Membrane Acidolysis (RMA) technology that uses the acidic environment of the late endosomes to weaken the membrane structure of the lipoplexes. This is achieved by a protonable basic position near the lipophilic part of the cationic lipids. Repulsive forces among the positively charged lipophilic parts of the lipids then ease the disruption of the endosomal membrane and the release of the genetic material. Alamar Blue dye (alamarBlue^{TM}) was purchased from Biosource International, Inc. (Camarillo, CA). The penicillin, streptomycin and L-glutamine solutions were obtained from the University of California San Francisco (UCSF) Cell Culture Facility (San Francisco, CA).

Plasmids

The plasmid pCMV.Luc (VR-1216; a gift of Dr. P. Felgner (Vical, San Diego, CA)) encoding luciferase was used for evaluating transfection efficiency under the control of the CMV promoter. The plasmid pSRVN.Luc-1430, encoding luciferase under the control of the human survivin promoter, which contains the proximal 1430 nt upstream of the survivin transcription start site [38-40], and two plasmids encoding luciferase under the control of the murine survivin promoter, pSRVN.Luc-1342 and pSRVN.Luc-194, were used for evaluating the survivin promoter-driven expression of luciferase. The promoter sequences in pSRVN.Luc-1342 are close to the full length of the survivin promoter, while pSRVN.Luc-194 is truncated at 194 bp upstream of the transcription initiation site [4]. Sharing an overall 84% identity with the human survivin, the mouse survivin protein contains a structurally unique single baculovirus *iap* repeat (BIR), that is required for apoptosis inhibition, and a –COOH-terminus coiled domain instead of a RING finger [4]. The plasmids were obtained from Dr. F. Li (Roswell Park Cancer Institute, Buffalo, NY) and propagated by Qiagen Inc. (Fargo, ND) (endotoxin level: <100 EU per mg).

Cell culture

Five human OSCC cell lines, two human non-tumor cell lines, one murine SCC cell line, and two murine non-tumor-derived cell lines were utilized in this study. Two cell lines derived from SCC of the tongue, HSC-3 [41] and H-357 [42],

were provided by Dr. R. Kramer (UCSF). H-413 cells, derived from SCC of the buccal mucosa, and H-376 cells, derived from SCC of the floor of the mouth [42], were obtained from Dr. R. Jordan (UCSF). The human oropharyngeal epidermoid carcinoma KB cell line [43] was obtained from the UCSF Cell Culture Facility. Non-tumor-derived human oral keratinocytes, GMSM-K, [44] were provided by Dr. V.A. Murrah (University of North Carolina at Chapel Hill). The 184A1 immortalized mammary epithelial cell line was derived from primary mammary cultures obtained from normal breast tissue and transformed *in vitro* with benzo(a)pyrene [45]. This cell line is not malignant when injected into nude mice, and it has a nearly complete diploid karyotype [46]. The cells were purchased from ATCC (CRL-8798, Rockville, MD). SCCVII, an aggressive murine cell line was established from the squamous cell carcinoma that developed spontaneously in C3H/HeJ mice and has been propagated subsequently *in vitro* [47]. For our studies, SCCVII cells were provided by Drs. D. Li and B. O'Malley (University of Pennsylvania, Philadelphia, PA). The NIH-3T3 mouse embryonic fibroblast continuous cell line, developed from a NIH Swiss mouse embryo [48], was obtained from the UCSF Cell Culture Facility. The NMuMG mouse mammary gland epithelial cell line, established through spontaneous immortalization of normal mammary epithelial cells of NAMRU mice [49], was purchased from ATCC (CRL-1636). The cells produce benign cystadenomas when inoculated into isogenic mice. The human cervical epithelial cancer cell line HeLa (ATCC) was also included in our study. These epithelial cells are readily transfectable and are used frequently in transfection assays.

All cell lines were maintained at 37°C in CO_2 (5%) incubators. Unless otherwise noted, all media were purchased from the UCSF Cell Culture Facility. HSC-3, H-357, H-376, HeLa, SCCVII and NIH-3T3 were cultured in Dulbecco's modified Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) (DMEM/10). H-413 cells were maintained in DMEM/10 supplemented with Ham's Nutrient Mixture F12. KB cells were cultured in Minimal Essential Medium (MEM) supplemented with 10% FBS. GMSM-K cells were propagated in keratinocyte-SFM medium supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Invitrogen, Carlsbad, CA). 184A-1 cells were maintained in serum-free Mammary Epithelial Base Medium (MEBM) supplemented with 5 ng/ml transferrin and 1 ng/ml cholera toxin (Clonetics, Walkersville, MD). NMuMG cells were grown in ATCC modified DMEM/10 medium containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 0.01 mg/ml bovine insulin and 10% FBS.

Transfection protocol

Cells were seeded in either 48-well or 24-well plates at a density between 1.0-3.0 x 10^5 cells per well in 1 ml of appropriate medium one day before the experiment, and used at approximately 60-80% confluence. The cells were pre-

washed with serum-free medium and then covered with 0.4 ml of the same medium. Lipid-DNA complexes were prepared by mixing Metafectene or Metafectene PRO with 100 µl of serum-free medium, followed by the addition of plasmid DNA. The mixture was incubated for 15 min at room temperature after the addition of the transfection reagent, and another 15 min after addition of DNA. Lipid/DNA complexes were added in a volume of 0.1 ml per well, the cells were incubated for 4 h at 37°C, and then 0.5 ml of serum-containing medium was added. The optimal ratio of Metafectene and Metafectene PRO to DNA was determined as described previously [50]. Luciferase activity was assayed 48 h after transfection, using the Luciferase Assay System (Promega, Madison, WI), and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The data were expressed as relative lights units (RLU) per ml of cell lysate or per mg protein. These values are designated "transfection activity". The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin Fraction V (Sigma) as the standard. The data obtained with the plasmids expressing luciferase under the control of the survivin promoter were normalized to the CMV promoter-driven expression. This comparison was used to account for the potentially different levels of transgene expression achieved in the different cell lines employed. To normalize transfection efficiency, we also used the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). In this system, the plasmid pRL-SV-40 that contains the *Renilla* luciferase gene under the control of the SV-40 promoter is co-transfected as an internal control. However, the highly variable Renilla luciferase expression in oral cancer cells made it difficult to use this system to normalize the survivin promoter-driven luciferase values.

Survivin ELISA

The level of survivin in cell lysates was measured using the human Total Survivin TiterZyme Enzyme Immunometric Assay (EIA) kit developed recently by Assay Designs Inc. (Ann Arbor, MI). This is a complete kit for the quantitative determination of human survivin in serum, plasma, urine, and cell lysates. Two-fold serial dilutions of recombinant human survivin, at a concentration range of 31.25-1000 pg/ml, were used as standards. The cell lysates were analyzed in duplicate, diluted 1:60 and 1:120, respectively. The standards and samples (100 µl/well) were incubated in plates pre-coated with a monoclonal antibody to survivin for 1 h at room temperature on a plate shaker at ~500 rpm. Subsequently, the plates were washed 5 times and 100 µl of rabbit polyclonal anti-survivin antibody was added to each well. The plates were incubated and washed as before, and 100 µl of a goat anti-rabbit IgG conjugated to horseradish peroxidase was added to each well. The plates were incubated for 30 min at room temperature, washed again and 100 µl of tetramethylbenzidine (TMB) substrate solution was added to each well. After 30 minutes, the reaction was stopped with 100 µl of 1 M HCl. The plate was read at 450 nm and 570 nm.

The concentration of survivin in cell lysates was determined by interpolation from a standard curve.

Cell viability assay

Cell morphology was evaluated by inverted phase contrast microscopy at 25x magnification. The number of viable cells used for the experiments was determined by Trypan Blue exclusion. Cell viability was quantified by a modified Alamar Blue assay [51, 52]. Cell viability (as a percentage of mock-treated control cells) was calculated according to the formula [(A_{570} - A_{600}) of test cells] x 100 / [(A_{570} - A_{600}) of control cells].

Statistical analysis

Data were compared for statistical significance by the unpaired Student's t-test, using StatView software (BrainPower, Inc., Calabasas, CA). A probability value (P) of less than 0.05 was considered significantly different.

RESULTS

Transgene expression in human cells driven by the survivin and CMV promoters

The ratios of transfection reagent/DNA (v/w) were selected based on the manufacturer's recommendations. At first, the transfection efficiency of Metafectene and Metafectene PRO was optimized in HeLa, HSC-3 and H413 cells as described previously [53]. Metafectene and Metafectene PRO were complexed with the pCMV.Luc plasmid at reagent:DNA ratios of 1 µl:0.5 µg, 2 µl:0.5 µg, 2 µl:1 µg or 4 µl:1 µg DNA. The optimal conditions for all three cell lines were 2 µl:1 µg DNA per well (Fig. 1A and Fig. 2A) and subsequent experiments were performed under these conditions. When compared to mock-transfected controls, the cytotoxic effect of the Metafectene- and Metafectene PRO-mediated transfection was very low (Fig. 1B and Fig. 2B). At 4 µl:1 µg DNA, Metafectene-mediated transfection was toxic in HSC-3 cells ($P<0.01$), while Metafectene PRO-mediated transfection was toxic in H413 cells ($P<0.05$). Metafectene was used at 2 µl:1 µg DNA (theoretical charge ratio of 3.68:1 (+/-)). To assess whether the activation of the survivin promoter is cancer-specific and to compare the cancer cell-specificity between the survivin promoter and the commonly used CMV promoter, tumor and non-tumor human cells were transfected with plasmids encoding luciferase under the control of either the human survivin promoter (pSRVN.Luc-1430) or the CMV promoter (pCMV.Luc) (Fig. 3A). A relatively high expression of luciferase activity driven by pSRVN.Luc-1430 was observed in HeLa cells (6,843 ± 2,607 RLU/ml), HSC-3 cells (1,720 ± 887 RLU/ml) and H376 cells (394 ± 202 RLU/ml). The two tumor cell lines, H357 and H413, did not display notably greater expression than that in the non-tumor-derived GMSM-K and 184A-1 cells. The activity of luciferase in H357 and H413 cells was not significantly different from that in

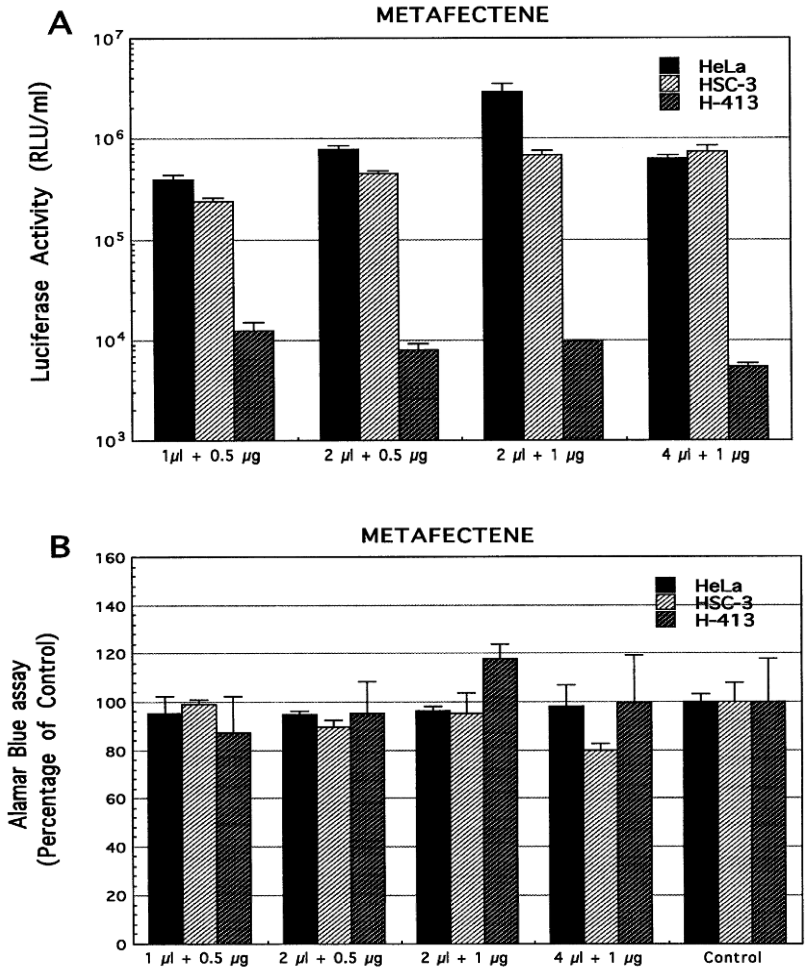

Fig. 1. Transfection activity and cytotoxicity of Metafectene under different conditions in HeLa, HSC-3 and H413 cells. A – Luciferase activity determined 48 h after transfection and expressed as relative light units (RLU) per ml of cell lysate. B – Cell viability measured by the Alamar Blue assay. Results are expressed as a percentage of mock-transfected controls. Data represent the mean ± S.D. obtained from triplicate wells.

GMSM-K cells ($P<0.375$). In all human cell lines, significantly higher luciferase activity was driven by the CMV promoter than by the human survivin promoter (Fig. 3). The highest expression of luciferase was observed in HeLa cells (341,900 ± 71,230 RLU/ml). The five OSCC cell lines displayed very different susceptibilities to transfection. The relatively high luciferase activities detected in H376, KB and HSC-3 cells were 131,683 ± 2,248, 62,560 ± 15,124 and 33,466 ± 2,953 RLU/ml, respectively. The expression of luciferase was much lower in H357 and H413 cells, 2,039 ± 856 and 2,048 ± 1,080, respectively. The results may be related to the less efficient internalization of lipoplexes by these

cells, since the survivin promoter-driven luciferase expression was also very low (~20 ± 10 RLU/ml). Overall the survivin promoter-driven luciferase expression was a small percentage of that driven by the CMV promoter. Notable among the cells were HSC-3 and HeLa for which the percentage were 5.1 and 2.0, respectively (Fig. 3B). The cells displayed very different susceptibilities to transfection; nevertheless, high CMV-driven luciferase activity appeared to correlate with high survivin-promoter driven luciferase expression.

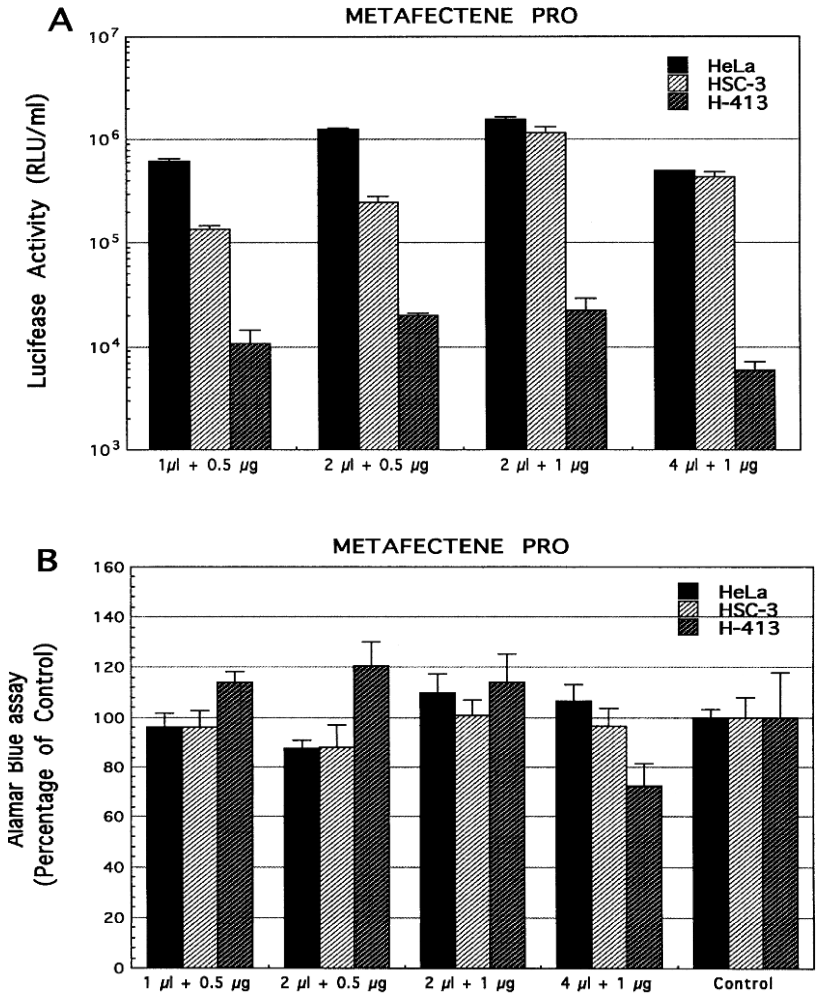

Fig. 2. Transfection activity and cytotoxicity of Metafectene PRO under different conditions in HeLa, HSC-3 and H413 cells. A – Luciferase activity determined 48 h after transfection and expressed as relative light units (RLU) per ml of cell lysate. B – Cell viability measured by the Alamar Blue assay. Results are expressed as a percentage of mock-transfected controls. Data represent the mean ± S.D. obtained from triplicate wells.

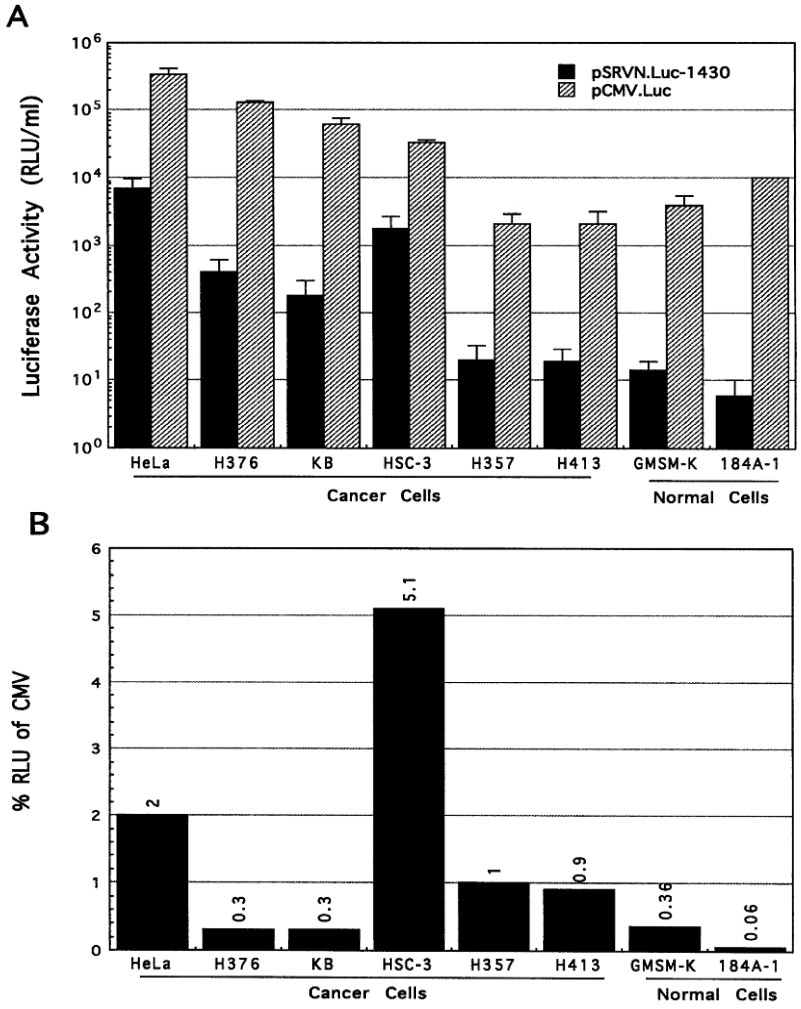

Fig. 3. The expression of luciferase by tumor and non-tumor human cells transfected with Metafectene-complexed plasmids encoding the enzyme under the control of either the human survivin promoter (pSRVN.Luc-1430) or the CMV promoter (pCMV.Luc). The cells were transfected with Metafectene at 2 µl/1 µg DNA. Results: A – as relative light units per ml (RLU/ml), B – as a percentage of CMV promoter-driven luciferase activity [(RLU induced by the survivin promoter/RLU induced by the CMV promoter) x 100%]. Values represent means ± S.D. and are representative of three separate experiments performed in triplicate. For details see Materials and Methods.

Transgene expression in murine cells driven by the survivin and CMV promoters

We next investigated the activity of luciferase expressed from plasmids encoding the enzyme under the control of two murine survivin promoters

(pSRVN.Luc-1342 and pSRVN.Luc-194) and the CMV promoter. We used a murine squamous cell carcinoma cell line, SCCVII, and two non-tumor-derived murine cell lines, 3T3-NIH and NMuMG (Tab. 1). SCCVII cells are employed in the generation of OSCC tumors in an oral cancer model in C3H/HeJ mice [34, 35]. Transfection efficiency of a novel polycationic transfection reagent, Metafectene PRO, was optimized in HeLa, SCCVII and NIH-3T3 cells. Metafectene PRO was used at 1 µl:0.5 µg, 2 µl:0.5 µg, 2 µl:1 µg, and 4 µl:1 µg DNA with the pCMV.Luc plasmid. The highest expression of luciferase was obtained with 2 µl Metafectene PRO:1 µg DNA [37], and the subsequent experiments were performed under this condition. When compared to mock-transfected controls, Metafectene PRO-mediated transfection with pCMV.Luc plasmid did not result in the reduction of total amounts of extractable cellular protein in lysates of HeLa, SCCVII and NIH-3T3 cells (data not shown).

The expression of luciferase driven by the CMV and survivin promoters in murine cells was much higher than that in human cells (Tab. 1). Significantly higher levels of luciferase activity were driven by the CMV promoter than by survivin promoters. SCCVII cells were readily transfectable [53]. The CMV promoter-driven, Metafectene PRO-mediated expression of luciferase in

Tab. 1. The expression of luciferase by SCCVII, 3T3-NIH and NMuMG cells transfected with Metafectene PRO-complexed with plasmids encoding the enzyme under the control of the murine survivin promoter (pSRVN.Luc-1342 and pSRVN.Luc-194) and the CMV promoter (pCMV.Luc).

Murine cells	Plasmid	Luciferase activity (RLU/ml)	% RLU of CMV
SCCVII	pCMV.Luc	10,733,667 ± 2,983,447	100.00
	pSRVN.Luc-1342	13,798 ± 670	0.13
	pSRVN.Luc-194	9,457 ± 1,201	0.09
NIH-3T3	pCMV.Luc	1,378,511 ± 20,513	100.00
	pSRVN.Luc-1342	7,069 ± 1,013	0.51
	pSRVN.Luc-194	2,095 ± 319	0.15
NMuMG	pCMV.Luc	5,257,333 ± 1,549,978	100.00
	pSRVN.Luc-1342	27,048 ± 13,555	0.51
	pSRVN.Luc-194	18,638 ± 1,662	0.35

The cells were transfected with Metafectene PRO at 2 µl/1 µg DNA. Results are shown as relative light units per ml (RLU/ml) or as a percentage of CMV promoter-driven luciferase activity [(RLU induced by the survivin promoter/RLU induced by the CMV promoter) x 100%] Values represent means ± S.D. and are representative of two separate experiments performed in triplicate. For details see Materials and Methods.

SCCVII cells was very high. The average value of luciferase activity (RLU/ml) was $10.7 \times 10^6 \pm 3.0 \times 10^6$, but in some experiments the values were even higher, in the range 20-36 x 10^6 RLU/ml. The high efficiency of transfection with the

pCMV.Luc plasmid was also observed in non-tumor-derived murine cells, NIH-3T3 and NMuMG (Tab. 1). The murine survivin promoters demonstrated greater transgene expression in murine cells than that observed in human cells with the human survivin promoter. The lowest expression of luciferase was found in the non-tumorigenic NIH-3T3 embryonic fibroblast cell line. Nevertheless, these values were still much higher than that obtained in human OSCC cells (Fig. 3A). The full survivin promoter (pSRVN.Luc-1342) produced consistently higher activity of luciferase compared to the truncated promoter (pSRVN.Luc-194). The highest expression of luciferase driven by the murine survivin promoters was observed in NMuMG cells (Tab. 1). These epithelial cells derived from the mammary gland, induce benign cystadenomas in isogenic mice and may have a potential for overexpression of survivin. In addition, this apparently normal cell population is capable to spontaneous malignant transformation in culture [54].

Correlation of the survivin promoter-driven luciferase expression with the survivin protein levels measured by the human survivin ELISA

All of the cancer cell lines derived from the epithelium were positive for survivin measured by ELISA (Tab. 2). The survivin protein concentration in cell lysates of cancer cells ranged from 5.8 ± 2.3 to 24.3 ± 2.9 ng per mg of protein (mean, 13.7 ng/mg). In H357 and H376 cells, in which the level of endogenous survivin was the lowest among the cells investigated, the survivin promoter-driven luciferase expression was also quite low. However, a comparison between HSC-3 and H376 cells indicates that the normalized (with respect to

Tab. 2. Metafectene-mediated expression of luciferase driven by the CMV promoter (pCMV.Luc) and the human survivin promoter (pSRVN.Luc-1430), and the levels of survivin protein in cell lysates of tumor and non-tumor human cells.

Cells	Luciferase activity (RLU/mg protein)[a]		Survivin (ng/mg protein)[b]
	pCMV.Luc	pSRVN.Luc-1430	
HeLa	268,677 ± 15,674	4,447 ± 2020	24.3 ± 2.9
KB	57,561 ± 15,231	192 ± 116	21.5 ± 0.8
HSC-3	52,551 ± 24,006	2,633 ± 919	12.0 ± 2.9
H413	1,024 ± 540	25 ± 16	12.6 ± 0.9
H357	1,731 ± 769	4 ± 2	6.2 ± 1.4
H376	61,275 ± 4,322	71 ± 38	5.8 ± 2.3
GMSM-K	1,721 ± 447	6 ± 2	16.7 ± 8.7
184A-1	4,031 ± 631	6 ± 6	13.5 ± 6.2

[a] The cells were transfected with Metafectene at 2 µl/1 µg DNA. Values represent means ± S.D. and are representative of three separate experiments performed in triplicate. [b] The level of survivin in cell lysates was measured using the human Total Survivin TiterZyme Enzyme Immunometric Assay. Survivin levels were normalized to total protein. Values represent means ± S.D. and are representative of two independent determinations. For details see Materials and Methods.

CMV-driven expression) survivin promoter-driven expression was 43-fold higher in HSC-3 cells, while the level of endogenous survivin was only 2-fold higher (Tab. 2). CMV-driven luciferase expression in HeLa cervical carcinoma cells was significantly higher that that in the oral cancer cells. However, the normalized survivin promoter-driven expression in HeLa cells (0.02) was lower than that in HSC-3 cells (0.05) (Fig. 3B), while the level of endogenous survivin was higher by a factor of 2 (Tab. 2). Thus, the efficiency of gene expression from transfected plasmid does not correlate with endogenous levels of survivin.

Interestingly, elevated survivin protein was determined in lysates of non-tumor-derived human cells. The values of ELISA survivin for GMSM-K oral keratinocytes and 184A-1 chemically immortalized, normal human mammary epithelial cells, were 16.7 ± 8.7 and 13.5 ± 6.2 ng/mg protein, respectively. It could be hypothesized that these immortal but not tumorigenic, transformed cell lines express all proteins, including survivin, which are necessary for proliferative activity. The high level of endogenous survivin did not correlate with high survivin-promoter driven luciferase expression in the same cells (Tab. 2).

DISCUSSION

Most viral and non-viral gene therapy approaches use non-specific viral promoters (CMV and SV40) that can be expressed at high levels even in non-tumor cells, potentially contributing to toxicity. An alternative approach involves transcriptional targeting that utilizes promoters activated preferentially in tumor cells, but not in normal cells. An ideal tumor-specific promoter (TSP) exhibits selective high activity in tumor cells (a "tumor on" phenotype). To diminish hepatotoxicity after systemic delivery, candidate promoters should display low activity in the liver (a "liver off" phenotype). Many TSPs have been explored for specific cancers, such as the prostate-specific antigen (PSA) for prostate cancer and the a-fetoprotein (AFP) promoter for hepatocarcinoma [55]. Recently, a novel TSP, the survivin promoter, that exhibits a tumor on/liver off phenotype in a wide range of tumors, has been described [12, 13]. To our knowledge, this is the first study that examined the survivin promoter-driven gene expression in oral cancer cells. These relatively comparable cells derived from OSCC tumors, displayed very different susceptibilities to transfection both with the survivin and the CMV promoter. In all cells, a significantly lower luciferase activity was driven by the survivin promoters than by the CMV promoter.

There are only a few reports describing the survivin promoter-driven gene expression mediated by liposomal vectors. Both the survivin promoter activities and the comparisons of their efficacies with that of viral promoters (CMV and SV40) have produced variable results. Although, the survivin promoter was usually more active in tumor cells than in non-tumor-derived cells, the tumor cells displayed very different susceptibilities to transfection [19, 21, 39, 56]. The activity of luciferase driven by the survivin promoter in cancer cells transfected with DOTAP:Chol liposomes was 10 to 40% of that driven by the CMV

promoter [19]. Conflicting results were also reported for the survivin vs. SV40 promoter activity in different cancer cells [19, 56].

Several groups have constructed conditionally replicating adenoviruses (CRAds), in which the survivin promoter regulates expression of the adenoviral early region 1A (*E1A*) gene [40, 55-58]. The transcriptional activity of the *survivin*-responsive CRAd in cancer cells was in the range of 1-16% of that driven by the CMV promoter [40]. To increase the transduction efficiency of *survivin*-responsive CRAds, Zhu *et al.* [55] incorporated a capsid modification (RGD or F5/3) into the adenovirus fiber region. The activity of luciferase driven by seven TSPs (Cox-2, CXCR4, EGP-2, HRP, SLPI, MsLn, and survivin) was evaluated in four human mesothelioma cell lines. The mean activity of luciferase for the survivin promoter was 8.9% of that for the CMV promoter. The adenoviral gene transduction efficiency was evaluated in cancer cells infected with E1-deleted replication-defective Ad.RSV-LacZ, Ad.CMV-LacZ and Ad.Surv-LacZ [58]. β-galactosidase activity depended both on cell type and the promoter. Ad.Surv-LacZ provided strong transcriptional activation in these cancer cells that display high β-gal activity after infection with Ad.CMV-LacZ.

In summary, the cancer-specificity of the survivin promoter appears greater than that of the CMV, SV40, and RSV promoters, but variations both in experimental conditions, and in presentations of the results (relative vs. absolute data), as well as extensive variability in the efficiency of transfection/transduction in different cancer cells make direct comparisons very difficult. There may be nucleotide sequence variation between survivin promoter clones isolated from different tumor cell lines. To evaluate this, we queried the Entrez Nucleotide Sequence Database producing six clones for the survivin/BIRC5 promoter. These clones were analyzed using BLAST sequence alignment producing no variation in sequence across the six clones. A few studies have investigated the survivin/BIRC5 promoter polymorphism. Xu *et al.* [59] described three mutations of the survivin/BIRC5 promoter. Mutations at the -235bp and -241bp sites were seen infrequently in the screened cell lines, which included tumor- and non-tumor-derived cells. In contrast, the C to G transition at -31bp has been observed frequently in cancer cells but not in normal cells tested. This finding was supported by Jang *et al.* [60], who described the polymorphism at -31bp as strongly correlating with the presence of lung cancer. Borbely *et al.* [61] refutes this finding indicating that the -31bp polymorphism does not strongly correlate with cervical cancer. This discrepancy in reporting indicates that the issue of polymorphisms in the survivin/BIRC5 promoter warrants continued investigation.

In an attempt to estimate whether the survivin promoter-driven luciferase expression is correlated to the level of endogenous survivin, we measured the amount of survivin protein in the lysates of cells that were used in transfection experiments. The survivin concentration in lysates of oral cancer cells ranged from 5.8 ± 2.3 to 21.5 ± 0.8 ng/mg protein (Tab. 2). These values are comparable to the levels of survivin (4.1 to 32.6 ng/mg protein) reported for

epithelial cell lines derived from prostate, bladder and breast tumors [62]. Survivin was undetectable in primary lymphocytes and in 8 of 10 tissue samples of non-tumor origin. Relatively low survivin content was found in samples taken from normal spleen and stomach [62]. The latter authors assume that these proliferative active tissues express all proteins that are necessary for correct chromosomal segregation. In our study, relatively high values of survivin were found in GMSM-K oral keratinocytes and 184A-1 human mammary epithelial cells. Our observation suggests a correlation between the expression of survivin and the proliferative activity of each cell type**.** Generally, cell lines that are not cancerous in origin must undergo some form of chemical immortalization to be cultured. We feel that this immortalization process may impact survivin production, making tumor/normal-tissue comparison by ELISA unrepresentative of wild-type conditions. Furthermore, ELISA data obtained from cultured cell lines will represent only the tissues that can be cultured. A low level of *survivin* mRNA was detected in normal WI-38 human fibroblasts and primary human osteoblasts [58]. By Western blot analysis, an intense survivin band was detected in transformed rat (ROSE-Tag, NuTu19, and NuTu26) and murine (IG10 and IF5) ovarian cell lines, but only a faint band was detected in early-passage normal ROSE and MOSE cells. A faint survivin band was detected in non-tumorigenic murine NIH-3T3 fibroblasts [19].

The observation that endogenous survivin expression does not correlate with luciferase expression from the exogenous survivin promoter suggests that the efficiencies of the endogenous and exogenous promoters are different. The level of endogenous survivin reflects the relatively high efficiency with which the chromosomal promoter mediates gene expression. The efficiency of the exogenous survivin promoter on the plasmid, transfected via a non-viral vector, appears to be compromised by the entire transfection process. The luciferase activity is a function of transfection efficiency. The expression of a transfected gene can suffer interference at any point from cell entry to post-translational modifications. This creates an inherent inaccuracy when comparing luciferase activity in different cell lines without a control for transfectional and expressional efficiency. Preliminary experiments with fluorescent Metafectene showed low levels of cell-associated fluorescence in cell types that expressed low luciferase activity (N. Düzgüneş, C. Lavoroni-Doyle, S. Gebremedhin and K. Konopka, unpublished data). Survivin-driven luciferase expression in oral cancer cells is much lower as a percentage ($\leq$ 5.1) of CMV-driven luciferase expression compared to other human cancer cells [21]. This observation is somewhat surprising in view of the fact that levels of endogenous survivin in oral cancer and other cancer cells are similar [62]. RT-PCR analyses showed that *survivin* mRNA is expressed in multiple cancer cells derived from a variety of tissue origins. The levels of *survivin* mRNA, however, varied widely among the different cancer cell lines, and not all cancer cells expressed a high level of survivin transcripts [9, 40, 58].

The inability of the survivin promoter to drive high-level gene expression in oral cancer cells appears to preclude its potential use in gene therapy. The rationale for the different susceptibility to transfection observed with different cells may include differences in binding, endocytosis and intracellular transport of liposome-DNA complexes, as well as in transcription activity. The rationale for the differences observed in endogenous survivin expression in oral cancer cells may include polymorphisms in the *survivin* gene promoter and/or variations in transcriptional as well as post-transcriptional regulation mechanisms. Future studies to elucidate the nature of these factors and their effects on transfection may enable the development of more effective therapy strategies.

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