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Inhibition of Epstein Barr Virus LMP1 gene expression in B lymphocytes by antisense oligonucleotides: Uptake and efficacy of lipid-based and receptor-mediated delivery systems

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Abstract

Epstein Barr Virus (EBV), is associated with an increasing number of lymphoid and epithelial malignancies. Among the genes expressed by EBV during latency, LMP1 plays a key role for growth transformation and immortalization of B lymphocytes. We have previously shown that antisense oligonucleotides (ONs) directed to LMP1 mRNA, effectively suppressed LMP1 gene expression and substantially reduced proliferation of the infected cells.

The use of antisense phosphodiester oligonucleotides as therapeutic agents is limited by inefficient cellular uptake and intracellular transport to the target mRNA. We tested the ability of three cationic carriers internalized by different pathways, to increase the delivery of anti-LMP1-ON to their site of action in EBV-infected B lymphocytes. We report here that liposomes, dendrimers or transferrin-polylysine-conjugated ON were internalized by the cells at an extent several fold higher than that of the naked oligomers. However, only the delivery system exploiting the transferrin receptor pathway of internalization, was able to vectorize biologically active antisense LMP1-ON.

Keywords: Antisense oligonucleotides; DNA vectors; LMP1; EBV; Lymphoma

1. Introduction

Epstein Barr Virus (EBV) is frequently associated with a variety of human malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma, T cell lymphoma, Hodgkin's disease and immunoblastic lymphoma in post-transplant and AIDS patients (Rickinson and Kieff, 2001). In addition, its infection immortalizes primary B lymphocytes *in vitro* into lymphoblastoid cell lines (Kieff and Rickinson, 2001; Miller et al., 1972). The virus often adopts latent forms in EBV-associated malignancies, expressing only a limited number of viral genes. Among the EBV latent gene products, LMP1 is the well-documented oncoprotein (Kieff and Rickinson, 2001; Li and Chang, 2003). This integral membrane protein is essential not only for EBV-induced immortalization of B lymphocytes, but expression of LMP1 alone can also cause transformation of rodent fibroblasts and epithelial cells *in vitro* (Kieff and Rickinson, 2001; Kim et al., 2000; Wang et al., 1985, 1988). In transgenic mice, LMP1 expression induces development of B cell lymphoma or epidermal hyperplasia (Kulwichit et al., 1998; Wilson et al., 1990). By functioning like a constitutively active receptor, LMP1 triggers multiple cell signaling events resulting in the up regulated expression of cellular genes involved in cell proliferation, cytokine secretion, angiogenesis, and tumor metastasis (Eliopoulos et al., 1999; Fries et al., 1996; Kim et al., 2000; Miller et al., 1995; Murono et al., 2001; Yoshizaki et al., 1998).

Synthetic antisense oligonucleotides (ONs) can be used to down-regulate the expression of specific genes. Their binding to a complementary sequence on an mRNA promotes the activation

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of RNase H endonucleases leading to degradation of the target transcript. For these reasons, they have been proposed as potent therapeutic agents for the treatment of important diseases such as oncogene-related cancers or viral infections (Hélène, 1991; Cohen, 1991; Crooke, 1992).

Because LMP1 plays a central role in EBV-mediated neoplastic transformation, we developed a 20-mer antisense LMP1 oligomer, named AS2, directed to the translation initiation site of LMP1 mRNA (Mattia et al., 1997). *In vitro* incubation of EBV-infected B95.8 cells with this oligomer caused a substantial loss of LMP1 mRNA, a significant decrease of LMP1 protein and a 50% inhibition of cell proliferation (Mattia et al., 1997). In view of a possible application of LMP1 antisense molecules in the treatment of those EBV-related lymphomas expressing LMP1 gene at high levels, we undertook studies aimed at increasing the intracellular delivery and availability of the AS2 oligomers by exploiting carriers with different uptake strategies.

In fact, cellular membranes are barriers for both hydrophilic and charged compounds as negatively charged antisense ONs, which have to be transported to and accumulate in sufficient concentration within the cytoplasm.

Therefore, we treated EBV-transformed B lymphocytes with AS2-ON vehicled by either lipid-based transfection reagents (TFX, Superfect (SF)) or bound to a ligand, like transferrin, internalized via receptor-mediated endocytosis.

We report here that LMP1 oligomers vehicled by either lipid-based vectors or by a transferrin polylysine (TFPL) complex, accumulated intracellularly more efficiently than the free ON. However, only AS2 oligomers internalized by transferrin-receptor were successfully released into the cytoplasm producing a rather complete suppression of LMP1 gene expression. This effect, obtained with a concentration of ON 20-fold lower than that used for the naked oligomers, was accompanied by a substantial decrement of cell proliferation. Surprisingly, among lipid-based vectors, SF-encapsulated AS2-ON, although vehicled at the highest extent, did not exert a biological effect because unavailable for interacting with the specific mRNA target.

2. Materials and methods

2.1. Cell culture

B95.8 cells, marmoset EBV-infected B lymphocytes (Rickinson and Kieff, 2001), were cultured in RPMI 1640 medium containing 2% (v/v) Biogro1 (Biological Industries), 1% penicillin–streptomycin. Cells were grown in a 5% CO_2 atmosphere at 37 °C and maintained at a cell density of 4×10^5 ml^{-1}.

2.2. Oligonucleotides

Unmodified 20-mer LMP1 antisense ON (AS2) targeting the translation initiation site of LMP1 mRNA and Sc2 ON, a scrambled version of LMP1 sequence (Mattia et al., 1997) were synthesized by MWG Biotechnology. To examine the uptake and distribution of antisense ON into B95.8 cells, 5′fluoresceinisothiocyanate-labeled AS2 oligomers (FITC-AS2) were used, which showed the same chemistry, length, base composition, purity and stability as the unlabeled ones. For uptake studies ONs were used in doses between 1 and 20 μM.

2.3. Transfection reagents and procedure

TFX®-50 (Promega, USA) is a mixture of a synthetic cationic lipid molecule [*N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis (2-hydroxyethyl)-2,3-di (oleoyloxy)-1,4-butanediammonium iodide] and L-dioleoyl phosphatidylethanolamine (DOPE). Superfect® (Qiagen, Germany) transfection reagent is a dendrimeric polycation of defined spherical architecture with branches radiating from a central core and terminating at charged amino groups.

PL-transferrinfection kit was purchased from Bender Medsystem (Burlingame, CA). Human transferrin was covalently linked to the small DNA-binding protein polylysine through a disulphide linkage. Transferrin-polycation molecules form stable complexes with DNA that can be internalized via Tf-receptor-mediated endocytosis.

Preparation of the reagent stock solutions, reagent/DNA mixtures and transfection procedures were performed, in principle, according to the Manufacturer's instructions.

2.4. Uptake studies

Exponentially growing B95.8 cells, seeded in 24-well plates (5×10^5 cells/well) were exposed for 2 or 4 h to increasing concentrations (0.5–20 μM) of fluoresceinisothiocyanate-labeled ON (FITC-ON), naked or complexed to the different carriers. After incubation, the cells were centrifuged three times (300 × g for 5 min); after each centrifugation step the pellet was washed with cold phosphate buffered saline solution (PBS). Cells were then fixed for 2 h at 4 °C in the dark, with either 2% formaldehyde or 70% ethanol at 10^6 cells ml^{-1} and analyzed by an Epics XL MCL cytofluorimeter (Coulter, Hialeah, FL)

2.5. Confocal laser scanning microscopy (CLSM) analysis

The cells were seeded onto 24-well plates at 4×10^5 ml^{-1} (10^5 cells/well). FITC-ONs, in the absence or presence of a carrier, were added to the cells at 20 μM and 1 μM, respectively, and incubated for 4 h at 37 °C. The cells were centrifuged at 300 × *g* for 5 min and washed three times with cold PBS before being fixed in a 4% formaldehyde buffered solution (Sigma) and analyzed using a Leica TCS 4D laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an Ar/Kr laser. FITC-ONs in the absence or presence of a carrier were excited at a wavelength of 488 nm, the fluorescence emission was collected after passing through long-pass filter of 510 nm. To minimize bleaching effect, the image acquisitions were made quickly on several cells presented on different slides for each sample and capturing signals from one field per slide. The Topography function was used to select specific intensity

data from an image stack obtained by a series of *xy*-sections. This function examines the sampling points (voxels) that are superimposed along the *z*-axis through all optical sections. The images represent orthogonal maximum projection obtained by calculating maximum intensity values. Acquisition parameters: objective 100.0/1.0 oil: pinhole size 50 Bm; pixel size 0.13 Bm, *z* pixel size 0.50 Bm, step size 0.50 Bm.

2.6. Proliferation rate of ON-treated B95.8 cells

B95.8 cells cultured to mid-log phase were diluted to 2.5×10^5 ml^{-1} and seeded in 24-well plates. At time 0, AS2 and Sc2 oligomers were added to the cells at 20 μM in the absence of carriers and at 1 μM if complexed with the transfection agents. At time 24 and 48 h, naked AS2 or Sc2 oligomers were added to all the cultures at a final concentration of 20 μM, independently of the delivery system used for the first addition. After 56 h of incubation, cells were harvested, counted in a hemocytometer and viability determined by Trypan blue dye exclusion. Cell counts were converted to percent of inhibition (Wickstrom et al., 1988).

2.7. Determination of LMP1 mRNA levels by RT-PCR

B95.8 cells were incubated with AS2 ON either free or complexed with the carriers as described above. After 56 h, total RNA was purified by Rneasy Mini Kit, Qiagen. RNA derived from about 120.000 cells was reverse-transcribed (gene Amp RNA PCR kit, Perkin-Elmer) in 20 μl volume, after the addition of 2.5 mM poly dT tailed primer. Ten microliters of the reverse transcription product were used to amplify LMP1 and gapdh with the specific primers under the conditions previously described (Masciarelli et al., 2002). Products of the amplification reactions were resolved in a 1.2% agarose gel and stained by ethidium bromide.

Gels were photographed and the negative images acquired in the computer by an Epson Perfection 1200 scanner. LMP1 mRNA specific signals were subjected to densitometric analysis using the Image J shared software (National Institutes of Health, MD).

2.8. Detection of intracellular RNA–DNA duplexes

Digoxigenin 3′ end-labeled AS2 ON, naked or complexed with either SF or TFPL conjugate according to the Manufacturer's instructions, were added to about 3.5×10^4 exponentially growing B95.8 cells at a final concentration of 1 μM. Unlabeled AS2 oligomers were added to the cells incubated with free oligomers to obtain a final concentration of 20 μM. After incubation for 4 h at 37 °C, cells were harvested, washed with 500 μl of pre-warmed (37 °C) PBS and resuspended in 200 μl of 150 mM ice-cold Hepes buffer pH 7.4. The cell suspension was lysed with 1/10 vol. of 5% Nonidet P40 in the presence of 20 U RNase inhibitor (Perkin-Elmer). After ethanol-precipitation, nucleic acids were resuspended in 100 μl of 0.05 M sodium acetate, pH 4.5, 0.28 M NaCl, 4.5 mM $ZnSO_4$. S1 nuclease assay was performed at 37 °C for 30′ in the presence of 30 U nuclease S1 (Promega). After addition of S1 stop mixture (0.5 M ammonium acetate, 25 mM EDTA containing 2 μg tRNA) and precipitation with ethanol, samples were resuspended in loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol), heated at 95 °C for 5 min, chilled on ice and then subjected to electrophoresis on a denaturing 7 M urea/10% polyacrylamide gel. Following transfer and UV binding on nylon membranes, labeled ONs were visualized by colorimetric detection (Roche Diagnostic Mannheim).

2.9. Determination of LMP1 protein levels by Western blotting

B95.8 cells, incubated for 56 h with LMP1 oligomers either free or complexed with TFPL according to the conditions described for the proliferation studies, were harvested by a 5 min centrifugation at 300 × *g* and washed with 500 μl PBS. Cell pellets were solubilized by sonication in 40 μl of sample buffer (Rowe et al., 1987) and boiled for 3 min. Protein concentration was determined by BioRad DC protein assay and 30 μg of each sample were subjected to electrophoresis on a 10% polyacrylamide/1% SDS gel. After blotting onto PVDF membranes immunological detection was carried out according to ECL Western Blotting system (Amersham Pharmacia Biotech). Primary antibodies, monoclonal anti-LMP1 (PharMingen) and anti-β-actin (Sigma), were used at a dilution of 1:20 000 and 1:1000, respectively. Secondary antibodies, peroxidase-labeled sheep anti-mouse Ig were used at a dilution of 1:5000. Blots treated with chemoluminescent reagents were exposed to Hyperfilm (Amersham Pharmacia Biotech).

3. Results

3.1. Cellular uptake of FITC-labeled AS2 oligomers

To optimize AS2-ON cellular delivery, we compared the uptake of free AS2-ON with that mediated by lipid-based carriers or by vectors internalized by receptor-mediated endocytosis.

FITC-labeled AS2 oligomers were complexed or not to the following vectors: (i) TFX reagents (TFX10®, TFX20® and TFX50®) are liposomes containing the same concentration of polycationic synthetic lipids but combined with different molar ratios of the fusogenic lipid DOPE; (ii) Superfect (SF), an activated dendrimeric transfection reagent; (iii) Transferrin-polylysine (TFPL) complex, as a carrier internalized via receptor-mediated endocytosis.

Free or vehicled FITC-AS2 oligomers, at concentrations ranging between 1 and 20 μM, were incubated with B95.8 cells for 4 h before measuring their uptake by flow-cytometry.

In Fig. 1, the values of mean fluorescence intensities (log) as functions of the different doses of ON are shown. It clearly appears that all carriers significantly increased cell-associated fluorescence in comparison to that determined after incubation with free FITC-AS2 oligomers. In fact, the fluorescence of the cells treated with FITC-AS2 oligomers, in the range between 1 and 10 μM, was similar to autofluorescence values of untreated cells, while only at 20 μM an increase of fluorescence could be

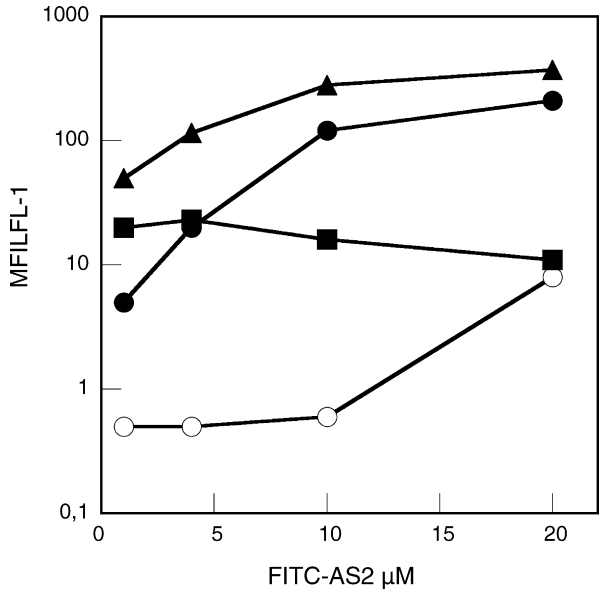

Fig. 1. Flowcytometric determinations of FITC-ON uptake by B95.8 cells. Cells were treated for 4 h with the indicated concentrations of FITC-labeled AS2-ON complexed to: SF (▲), TFX (●), TFPL (■) or not complexed (◯). Thereafter the cells were washed, fixed and analyzed in a flow-cytometer. For each sample, at least 10 000 events were collected and the log of mean fluorescence intensities were recorded. The cells not receiving FITC-labeled-ON represented the autofluorescence, which was identical to the lowest fluorescence of FITC-AS2-ON. Here, one representative experiment out of three performed is shown.

detected. Lipid-based carriers delivered the ON to a much higher extent than the TFPL complex, with the SF reagent being more effective than TFX, especially for the two lower concentrations of FITC-AS2. Moreover, in several experiments, the different TFX formulations were equally effective, independently of the internal ratio of the lipidic components (data not shown). Surprisingly, while the uptake mediated by TFX and SF increased with ON concentrations and reached a plateau at 10 μM, the uptake mediated by TFPL was maximal already at the lowest concentration (1 μM) of FITC-AS2 oligomer and gradually decreased for higher amounts of complexed ON.

3.2. Intracellular localization of AS2-ON by confocal microscopy

To study the intracellular localization of the labeled ON, the distribution of either naked or vector-delivered FITC-AS2 oligomers was studied by confocal laser scanning microscopy analysis (CLSM). Free oligomers were detectable only at a concentration of 20 μM while ON complexed to TFX, SF and TFPL carriers were used at a concentration of 1 μM.

The images in Fig. 2a illustrate the overall fluorescence of B95.8 cells after incubation with either free or complexed FITC-ON, while the images in Fig. 2b represent single optical sections in which the distribution patterns of the AS2 oligomers inside the cells were analyzed. From the comparison of the images it appears that the labeled oligomers distribute within B95.8 cells differently, depending on the uptake system by which they have been internalized. In general, free AS2-ON and ON complexed

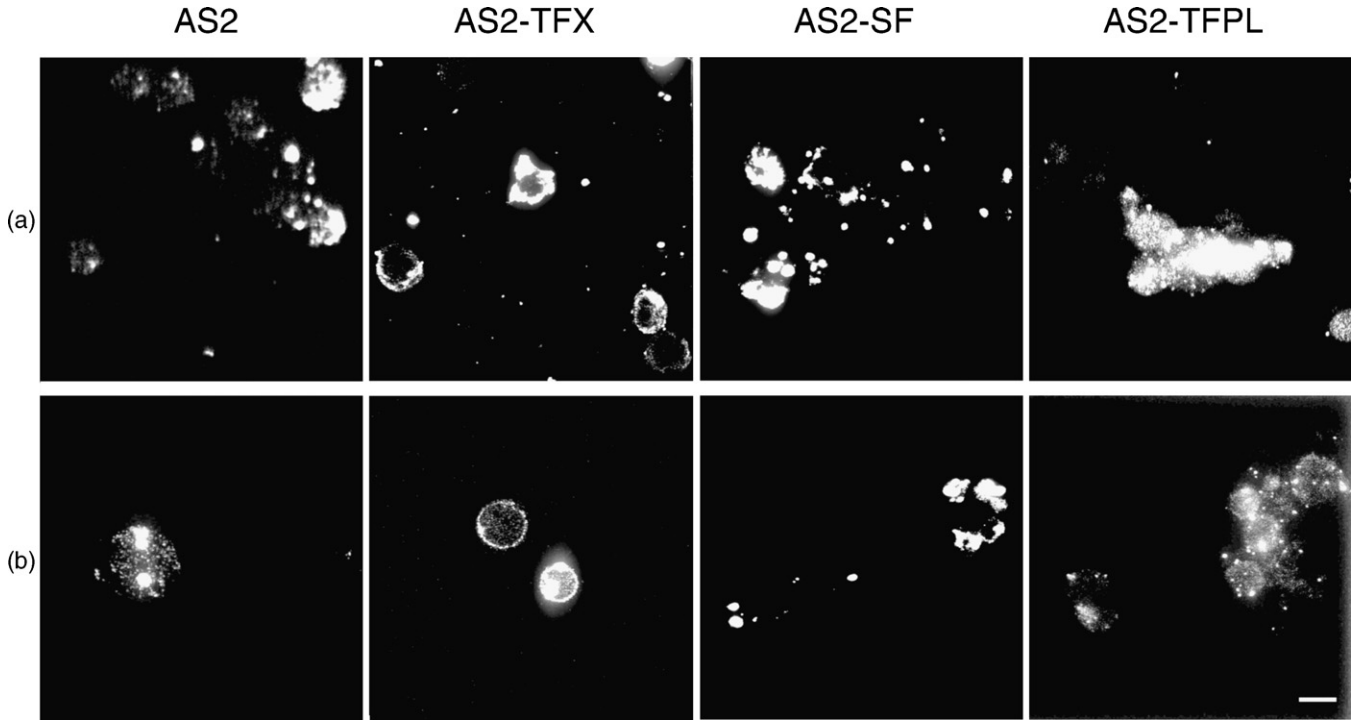

Fig. 2. Intracellular uptake of FITC-AS2 oligomers into B95.8 cells with or without complexation to carriers. B95.8 cells were incubated for 4 h with FITC-AS2 either free or bound to the different vectors, fixed and analyzed with a laser confocal microscope. Pannels in (a) orthogonal maximum intensity projections obtained by 10 sections. Pannels in (b) mean optical sections of the treated cells. AS2: uptake of free FITC-AS2 at 20 μM; AS2-SF, AS2-TFX, AS2-TFPL: uptake of FITC-AS2 at 1 μM complexed to SF, TFX, or TFPL, respectively. The scale bar represents 10 μm.

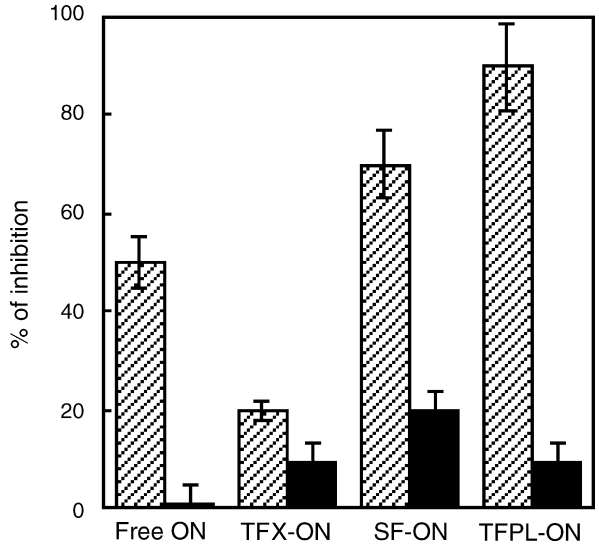

Fig. 3. Growth ihibition of B95.8 cells incubated with AS2 or Sc2 ON either free or vectorized. B95.8 cells (2.5×10^5 ml^{-1}) were incubated with LMP1 ON (AS2 and Sc2) either free at 20 μM (naked ON) or vehicled at 1 μM (ON-TFX, ON-SF, ON-TFPL), as described in Section 2. At 24 and 48 h, 20 μM of naked AS2 or Sc2 oligomers were added to all cultures. Cells were harvested after a total incubation period of 56 h. Cell counts were assessed by trypan blue exclusion and the percent inhibition of proliferation was calculated. Hatched bars: cells treated with AS2-ON; filled bars: cells treated with Sc2-ON. Values and bars are mean ± S.D.

to TFPL, produced a finely punctuated pattern inside the cells, while AS2-ON vehicled by the lipid-based carriers appeared non homogeneously internalized by the lymphocytes with cells weakly labeled and cells showing highly fluorescent patches of different size. Single optical sections analysis (Fig. 2b) revealed that, in most of the cells, independently of the delivery system, the nuclei were excluded from the labeling. In particular, TFX-complexed ON, attached to the cells as large agglomerates, appeared to be internalized as small vesicles, detectable at the periphery of the cell. In addition, cells treated with SF-ON were altered in shape with fluorescence spots both inside and outside the cells. Interestingly, cell-associated fluorescence of TFX-ON used at the concentration of 1 μM was much lower than that detected in B95.8 cells after treatment with the same concentration of TFPL-complexed ON.

3.3. Effect of vehicled AS2-ON on B95.8 cell proliferation

Proliferation rate of cells treated with vehicled AS2 oligomers was compared to that of cells incubated with the free ON. A non-sense LMP1 sequence, termed Sc2 (a randomly scrambled version of the AS2 sequence), was used as a control. Preliminary experiments carried out to evaluate spontaneous activation of EBV lytic cycle in untreated and ON-treated cells, indicated that incubation with the oligomers did not increase the percentage of B95.8 cells that lyse, independently of the type of carrier and/or the length of the treatment.

Fig. 3 shows that treatment of B95.8 cells with 20 μM of free AS2 oligomers resulted in a 50% inhibition of cell proliferation, confirming the data previously reported (Mattia et al., 1997). When AS2 oligomers were delivered to the cells by SF or by TFPL at a concentration 20 times lower, cell proliferation was inhibited by 70% and 90%, respectively. Unexpectedly, TFX-ON inhibited B95.8 cell proliferation only by 20%, an extent even lower than that reached by incubating the cells with the naked AS2 oligomers. Control experiments carried out with Sc2-ON indicated that cell growth was not inhibited when these oligomers were administered free to the cells and was slightly inhibited when they were complexed to TFX or TFPL. However, between 10 and 30% inhibition was observed when the control ONs were vehicled by SF. This result correlated with an increased number of trypan blue stained cells indicating some degree of toxicity of this carrier for B95.8 cells.

Because TFX delivered ON inside B95.8 cells less efficiently than SF, reducing cell proliferation only by 20%, the following experiments were conducted with SF and TFPL vectors.

3.4. LMP1 mRNA levels after treatment of B95.8 cells with naked or vehicled antisense ON

To investigate whether the stronger inhibition of cell proliferation observed after treating B95.8 cells with complexed AS2-ON was dependent on a more effective down-regulation of LMP1 expression, mRNA levels were measured by RT-PCR experiments and compared to those detected in cells exposed to free LMP1 antisense oligomers.

Fig. 4 illustrates the results of RT-PCR analysis conducted on B95.8 cells incubated for 56 h with free or complexed AS2-ON. As previously reported (Mattia et al., 1997), Fig. 4a shows that treatment of B95.8 cells with uncoupled AS2-ON resulted

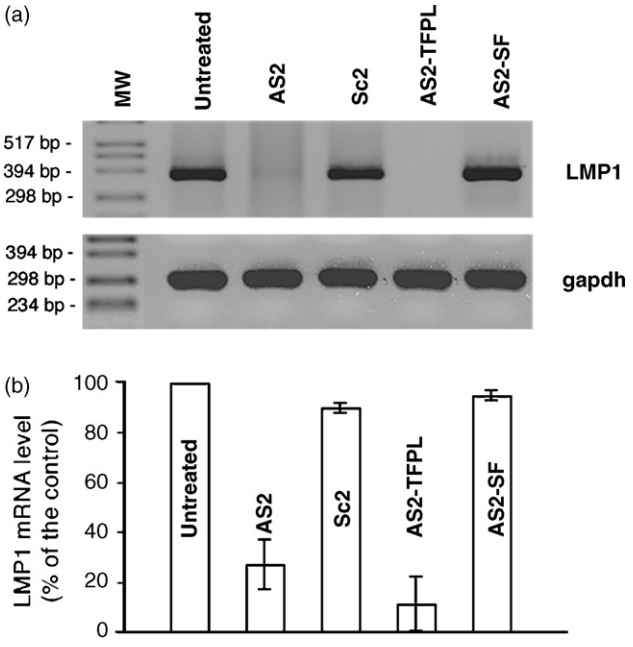

Fig. 4. Analysis of LMP1 transcripts in B95.8 cells untreated and treated with free or vehicled LMP1 oligomers. B95.8 cells were incubated as described in Fig. 3. Total RNA was extracted, reverse transcribed and used for amplification of LMP1 and gapdh cDNA (see Section 2). (a) PCR products were separated on a 1.2% agarose gel and stained by ethidium bromide; (b) quantification of the specific signals by densitometry. Each value of the bar graph represents the mean of three independent experiments ± S.D.

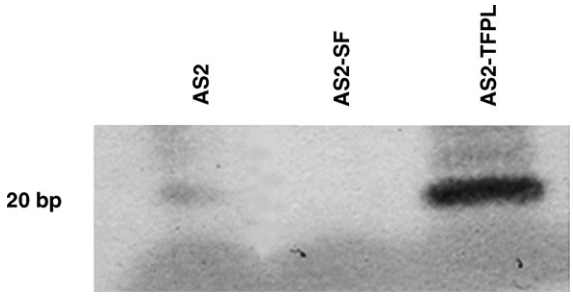

Fig. 5. Detection of intracellular DNA-RNA duplex in B95.8 cells cultured in the presence of free or complexed AS2-ON. B95.8 cells were incubated for 4 h with digoxigenin-labeled AS2-ON administered with or without SF or TFPL vectors. Cell suspension was lysed and ethanol-precipitated. After resuspension of nucleic acids, S1-nuclease assay was carried out for 30 min at 37 °C, as described in Section 2. Following a further precipitation, samples were resolved on a 7 M urea/10% polyacrylamide gel, transferred to nylon membranes and subjected to colorimetric detection.

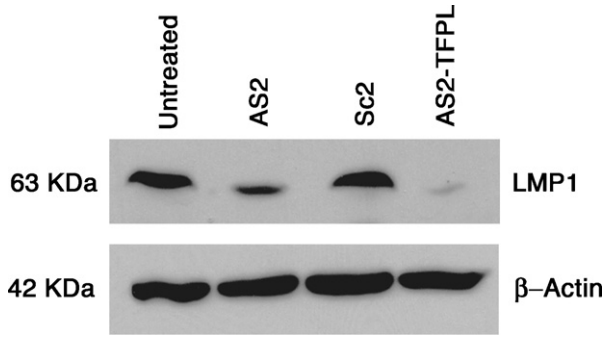

Fig. 6. Immunoblot determination of LMP1 protein levels in B95.8 cells exposed to free AS2-ON or to AS2-TFPL complex. B95.8 cells were treated as described for Fig. 3. For each sample, 30 μg of cell lysate were electrophoresed and transferred to membranes. Blots were incubated with monoclonal LMP1 and β-actin primary antibodies followed by peroxidase labeled anti-mouse Ig. Positive signals were visualized by chemoluminescent detection.

in a marked down regulation of LMP1 mRNA levels as compared to those detected in untreated cells or in cells exposed to the control Sc2-ON. However, a consistently larger suppression of the LMP1 mRNA signal was observed when TFPL was used as a vector. The results of the densitometric analysis of the LMP1 mRNA signals derived from three independent experiments and reported in Fig. 4b, indicated that the specific messenger was reduced by about 75% when AS2-ON was administered free to the cells and by about 85% when vehicled by TFPL. Surprisingly, transfection with SF-AS2 complex did not affect LMP1 mRNA levels that remained similar to those detected for untreated or Sc2-treated cells.

3.5. Intracellular DNA-RNA duplex formation in B95.8 cells after delivery of AS2 oligomers

To understand why LMP1 mRNA levels were not affected when B95.8 cells were treated with SF-AS2, intracellular duplex formation was evaluated after incubation with digoxigenin-labeled AS2 oligomers either free or delivered by SF and TFPL.

After RNA isolation and digestion by S1 nuclease, heteroduplex formed by labeled antisense oligomers and LMP1 mRNA target sequence, were detected as described in Section 2. Fig. 5 shows that a weak signal was obtained in the lane corresponding to B95.8 cells exposed to naked antisense ON. However, a signal about 50-fold stronger was observed in the sample representing the cells exposed to AS2-TFPL. This result confirmed the higher uptake of the AS2-TFPL complex with respect to the free AS2 oligomers and the full capability for the vectorized ON to interact with the target LMP1 mRNA. In contrast, hybrid RNA/DNA formation was not detected in the sample corresponding to B95.8 cells incubated with AS2-SF clearly indicating that AS2 oligomers delivered to the cytoplasm by the dendrimeric vector, were unable to bind the specific mRNA sequence.

3.6. LMP1 protein levels in B95.8 cells treated with TFPL-conjugated AS2 ON

To further evaluate the biological activity of AS2-TFPL internalized by B95.8 via receptor-mediated endocytosis, LMP1 protein levels were studied after incubation of the cells with AS2 oligomers either free or coupled to the TFPL. Fig. 6 illustrates the results obtained by immunoblot analysis. LMP1-specific monoclonal antibodies detected a 63 kDa protein band of equal intensity in the lysate of untreated B95.8 cells or after incubation with control Sc2 oligomer. In the lane corresponding to the cells exposed to AS2-ON, LMP1 signal decreased by about 60%. More strikingly, the LMP1 band was hardly detectable in the cells exposed to AS2 oligomers complexed to TFPL. This result confirmed that the LMP1 antisense molecules delivered by this carrier, were biologically active and able to produce a rather complete suppression of LMP1 expression. Moreover, Sc2 oligomers delivered by TFPL had no effect on the amount of LMP1 protein which remained similar to that observed in B95.8 cells incubated with the same oligomer in the absence of the vector (data not shown).

4. Discussion

Although the clinical relevance of ONs for the treatment of a wide variety of diseases, such as cancer and viral diseases has been demonstrated (Wang et al., 2001), poor stability and inefficient cellular uptake, both *in vitro* and *in vivo*, limit the efficacy of ON and have been a barrier to therapeutic development. Cellular uptake, depending on ONs chemistry and cell type, may be less than 2% of the dose (Lucas et al., 2005). As a result, ON concentration at the active site, especially in terms of *in vivo* drug delivery, may be too low for an effective and sustained outcome. Indeed, a majority of the current and past research focuses on the improvement of ON stability and delivery through modification of the ON structure itself (Zamaratski et al., 2001; Dean and Bennett, 2003; Tarkanyi et al., 2005; Liu and Huang, 1989) or via the use of vectors, which can both protect the drug from degradation as well as improve ON delivery inside the cells.

While the presence of EBV is found in an increasing number of malignancies, a strong correlation between virus infection and secondary lymphomas originated in post-transplanted and immuno-depressed patients, is well-documented (Gottschalk et al., 2005; Heslop, 2005).

In view of the possibility for using LMP1 oligomers as therapeutics in EBV-associated tumors, we undertook studies meant to evaluate the uptake mediated by different delivery systems and the availability of the internalized antisense molecules for interacting with the target mRNA sequence.

The most widely used liposomes, composed from artificial cationic lipids which associate and neutralize negatively charged ONs, enter the cells by endocytosis. The addition of the fusogenic lipid DOPE to the liposome formulation helps the destabilization of endosomal membrane favoring the release of ON to the site of action. Although TFX-50 is a liposome preparation formulated with a high molar ratio of DOPE to cationic lipids, this vector was probably unable to successfully release the antisense into the cytoplasm of B95.8 cells. In fact, despite the increment of ON uptake obtained with this liposomal vector, inhibition of cell proliferation was even lower than that achieved by treating the cells with naked oligomers.

Recently, new input in research investigating the potential of dendrimers as ON and/or DNA delivery vehicles has occurred (Yoo and Juliano, 2000; Sato et al., 2001). The highly reproducible complex formation between surface functional groups and ON, the weak cytotoxicity and enhanced delivery even in the presence of high concentrations of serum, has indicated a possible advantage of dendrimers over liposomes. By using this system we have obtained the highest intracellular concentration of ON as measured by both cytofluorimetric as well as by confocal microscopy analysis.

Nevertheless, despite the efficient uptake of the SF-ON complexes and the inhibitory effect on cell growth, the lack of intracellular mRNA-ON duplex formation and of LMP1 mRNA degradation, indicate a failure, also of this carrier, to successfully release the antisense oligomers intracellularly. In addition, the significant decrement of cell proliferation observed with the control Sc2 oligomer and the large fluorescent vesicles found both inside and outside the cells treated with AS2-SF, strongly suggest some toxicity of the positively charged dendrimers on the lymphocytes' membranes. It is therefore likely that cell growth inhibition determined in B95.8 cells exposed to AS2-SF complex might be due in part to vector toxicity and in part to free AS2-ON added to the cell culture during the time frame of the experiment.

An attractive strategy to specifically deliver ON to defined target cells is the use of import mechanisms already present in the cell membrane for the uptake of biomolecules necessary for cell function. It has been reported that LMP1 expression up-regulates the transferrin receptor number in EBV-infected B lymphocytes (Wang et al., 1988). The transferrin receptor endocytic pathway could therefore not only improve ON internalization, but also mediate targeted delivery to virus-harboring cells that over express the transferring receptor.

We report here that LMP1 antisense ONs delivered by TFPL conjugate, efficiently hybridized to target mRNA and dramatically improved the biological effect of free AS2 molecules causing a complete suppression of LMP1 gene expression and a 90% inhibition of B95.8 cell proliferation. Remarkably, these effects were determined with a concentration of antisense oligomers 20 times lower than that effective with naked AS2. In this respect we also observed that increasing concentration of AS2 complexed to TFPL were in fact decreasing the uptake of the ON, suggesting that the antisense oligomers might hinder receptor-ligand binding by masking receptor-binding sites on transferrin.

The efficacy of this vector is based on the higher uptake of the AS2-TFPL with respect to that of naked AS2 oligomers, and, most of all, on the availability of the internalized antisense molecules for binding with the target LMP1 mRNA. In fact, both liposomes and dendrimers increased the uptake of AS2-ON to an extent even higher than that determined by TFPL vector. In this respect, a possible contribution of the lipophilic nature of the FITC moiety in promoting AS2 delivery by liposomal vectors cannot be completely ruled out. However, our results showing similar uptake values for ON complexed to TFX preparations containing different molar ratio of the fusogenic lipid DOPE, seem to exclude this possibility. Therefore, the lack of biological activity of the oligomers delivered to B95.8 cells by lipid carriers, indicates that AS2-ON is impeded to reach the site of action in the cytoplasm.

TFX and SF are commercially available transfection reagent, commonly used for delivering DNA to the cells. The DNA-carrier complexes require endosomal and/or lysosomal fusion in order to dissociate, following acidification of the compartments (Schmidt-Wolf and Schmidt-Wolf, 2003). It should be considered, however, that the interactions of small single stranded ONs with lipid-based cationic carriers might be much stronger than those occurring with plasmid, double stranded DNA. In addition, CLSM images show that liposomal and dendrimeric complexes tend to form large aggregates. It seems conceivable that antisense ONs remains very tightly bound to liposomes or dendrimers despite the pH changes occurring in the endosomal pathway. This hypothesis is supported by recent fluorescent fluctuation spectroscopy (FFS) studies that, investigating the complexation (association and dissociation) of ON and cationic liposomes in Vero cells, have clearly shown that the major part of the Cy5-ON remained complexed to FITC-liposome, and that the complexes seemed to aggregate (Lucas et al., 2005).

To this regard, the finely punctuated regions observed in the cytosol of B95.8 cells treated with FITC-AS2-TFPL complexes, similar to those observed when naked FITC-AS2 oligomers are internalized, could be an indication of occurred dissociation of the ON from the TFPL carrier.

TFPL conjugates have been shown to be efficient carriers for gene delivery into many leukemic and hematopoietic cells (Citro et al., 1992; Zenke et al., 1990), and systemic application of transferrin-polyethylenimine DNA complexes resulted in successful expression of the delivered gene in distant tumors (Kircheis et al., 2001). However, although the pathway of transferrin receptor-mediated endocytosis is well characterized, the modalities by which DNA bound to TFPL is released from the carrier and gets access to the cytoplasm are not completely understood, and different strategies have been developed to ensure the exit of the endocytosed material from the endosome (Zauner et al., 1998).

This study has revealed that TFPL represents a suitable system for delivery of LMP1 antisense ON to lymphocytes.

Preliminary experiments, carried out on EBV-infected cell lines of different origin, have shown that TFPL-delivered antisense LMP1 efficiently reduced proliferation of those lines harboring a LMP1 target sequence fully complementary to the AS2 oligomers.

Considering that full biological activity is obtained with only one 20th of the AS2 concentration needed to down-regulate LMP1 expression in the absence of a vector, and the negligible level of cytotoxicity, TFPL-vectorized ONs should be used for further studies aimed to assess the therapeutic potential of LMP1 antisense ON in EBV-related lymphomas expressing high levels of the viral oncogene.

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References

Citro, G., Perrotti, D., Cucco, C., D'Agnano, I., Sacchi, A., Zupi, G., Calabretta, B., 1992. Inhibition of leukemia-cell proliferation by receptor-mediated uptake of C-myb antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. U.S.A. 89, 7031–7035.

Cohen, J.S., 1991. Oligonucleotides as therapeutic agents. Pharmacol. Ther. 52, 211–225.

Crooke, S.T., 1992. Therapeutic applications of oligonucleotides. Biotechnology (NY) 10, 882–886.

Dean, N.M., Bennett, C.F., 2003. Antisense oligonucleotide-based therapeutics for cancer. Oncogene 22, 9087–9096.

Eliopoulos, A.G., Gallagher, N.J., Blake, S.M., Dawson, C.W., Young, L.S., 1999. Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. J. Biol. Chem. 274, 16085–16096.

Fries, K.L., Miller, W.E., Raab-Traub, N., 1996. Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. J. Virol. 70, 8653–8659.

Gottschalk, S., Rooney, C.M., Heslop, H.E., 2005. Post-transplant lymphoproliferative disorders. Annu. Rev. Med. 56, 29–44.

Hélène, C., 1991. Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides. Eur. J. Cancer 27, 1466–1471.

Heslop, H.E., 2005. Biology and treatment of Epstein-Barr virus-associated non-Hodgkin lymphomas. Hematology, 260–266.

Kieff, E., Rickinson, A.B., 2001. Epstein-Barr virus and its replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 2511–2573.

Kim, K.R., Yoshizaki, T., Miyamori, H., Hasegawa, K., Horikawa, T., Furukawa, M., Harada, S., Seiki, M., Sato, H., 2000. Transformation of Madin-Darby canine kidney (MDCK) epithelial cells by Epstein-Barr virus latent membrane protein 1 (LMP1) induces expression of Ets1 and invasive growth. Oncogene 19, 1764–1771.

Kircheis, R., Wightman, L., Schreiber, A., Robitza, B., Rossler, V., Kursa, M., Wagner, E., 2001. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther. 8, 28–40.

Kulwichit, W., Edwards, R.H., Davenport, E.M., Baskar, J.F., Godfrey, V., Raab-Traub, N., 1998. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 95, 11963–11968.

Li, H.P., Chang, Y.S., 2003. Epstein-Barr virus latent membrane protein 1: structure and functions. J. Biomed. Sci. 10, 490–504.

Liu, D.X., Huang, L., 1989. Small, but not large, unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid can be stabilized by human plasma. Biochemistry 28, 7700–7707.

Lucas, B., Remaut, K., Sanders, N.N., Braeckmans, K., De Smedt, S.C., Demeester, J., 2005. Towards a better understanding of the dissociation behavior of liposome-oligonucleotide complexes in the cytosol of cells. J. Control Rel. 103, 435–450.

Masciarelli, S., Mattioli, B., Galletti, R., Samoggia, P., Chichiarelli, S., Mearini, G., Mattia, E., 2002. Antisense to Epstein Barr virus-encoded LMP1 does not affect the transcription of viral and cellular proliferation-related genes, but induces phenotypic effects on EBV-transformed B lymphocytes. Oncogene 21, 4166–4170.

Mattia, E., Chichiarelli, S., Hickish, T., Gaeta, A., Mancini, C., Cunningham, D., van Renswoude, J., 1997. Inhibition of in vitro proliferation of Epstein Barr virus infected B cells by an antisense oligodeoxynucleotide targeted against EBV latent membrane protein LMP1. Oncogene 15, 489–493.

Miller, G., Shope, T., Lisco, H., Stitt, D., Lipman, M., 1972. Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. 69, 383–387.

Miller, W.E., Earp, H.S., Raab-Traub, N., 1995. The Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor. J. Virol. 69, 4390–4398.

Murono, S., Inoue, H., Tanabe, T., Joab, I., Yoshizaki, T., Furukawa, M., Pagano, J.S., 2001. Induction of cyclooxygenase-2 by Epstein-Barr virus latent membrane protein 1 is involved in vascular endothelial growth factor production in nasopharyngeal carcinoma cells. Proc. Natl. Acad. Sci. U.S.A. 9, 6905–6910.

Rickinson, A.B., Kieff, E., 2001. Epstein-Barr virus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 2575–2627.

Rowe, M., Evans, H.S., Young, L.S., Hennessy, K., Kieff, E., Rickinson, A.B., 1987. Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. J. Gen. Virol. 68, 1575–1586.

Sato, N., Kobayashi, H., Saga, T., Nakamoto, Y., Ishimori, T., Togashi, K., Fujibayashi, Y., Konishi, J., Brechbiel, M.W., 2001. Tumor targeting and imaging of intraperitoneal tumors by use of antisense oligo-DNA complexed with dendrimers and/or avidin in mice. Clin. Cancer Res. 7, 3606–3612.

Schmidt-Wolf, G.D., Schmidt-Wolf, I.G., 2003. Non-viral and hybrid vectors in human gene therapy: an update. Trends Mol. Med. 9, 67–72.

Tarkanyi, I., Horvath, A., Szatmari, I., Eizert, H., Vamosi, G., Damjanovich, S., Segal-Bendirdjian, E., Aradi, J., 2005. Inhibition of human telomerase by oligonucleotide chimeras, composed of an antisense moiety and a chemically modified homo-oligonucleotide. FEBS Lett. 579, 1411–1416.

Wang, D., Liebowitz, D., Kieff, E., 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell 43, 831–840.

Wang, D., Liebowitz, D., Wang, F., Gregory, C., Rickinson, A., Larson, R., Springer, T., Kieff, E., 1988. Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. J. Virol. 62, 4173–4184.

Wang, H., Prasad, G., Buolamwini, J.K., Zhang, R., 2001. Antisense anticancer oligonucleotide therapeutics. Curr. Cancer Drug Targets 1, 177–196.

Wickstrom, E.L., Bacon, T.A., Gonzalez, A., Freeman, D.L., Lyman, G.H., Wickstrom, E., 1988. Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-myc mRNA. Proc. Natl. Acad. Sci. U.S.A. 85, 1028–1032.

Wilson, J.B., Weinberg, W., Johnson, R., Yuspa, S., Levine, A.J., 1990. Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. Cell 61, 1315–1327.

Yoo, H., Juliano, R.L., 2000. Enhanced delivery of antisense oligonucleotides with fluorophore-conjugated PAMAM dendrimers. Nucl. Acids Res. 28, 4225–4231.

Yoshizaki, T., Sato, H., Furukawa, M., Pagano, J.S., 1998. The expression of matrix metalloproteinase 9 is enhanced by Epstein-Barr virus

latent membrane protein 1. Proc. Natl. Acad. Sci. U.S.A. 95, 3621–3626.

Zamaratski, E., Pradeepkumar, P.I., Chattopadhyaya, J., 2001. A critical survey of the structure-function of the antisense oligo/RNA heteroduplex as substrate for RNase H. J. Biochem. Biophys. Meth. 48, 189–208.

Zauner, W., Ogris, M., Wagner, E., 1998. Polylysine-based transfection systems utilizing receptor-mediated delivery. Adv. Drug Deliv. Rev. 30, 97–113.

Zenke, M., Steinlein, P., Wagner, E., Cotton, M., Beug, H., Birnstiel, M.L., 1990. Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. Proc. Natl. Acad. Sci. U.S.A. 87, 3655–3659.