Different effects of an oligonucleotide uptake stimulating protein on leukemic cells in their primitive and differentiated state

György Tóth[a,*], József Schlammadinger[a], Sándor Sipka[c], Attila Kiss[b], Gyula Szegedi[c], Kálmán Rák[b], Gábor Szabó[1,a]

[a] *Department of Biology, University Medical School of Debrecen, P.O. Box 1, H-4012 Debrecen, Hungary*
[b] *2nd Department of Internal Medicine, University Medical School of Debrecen, P.O. Box 1, H-4012 Debrecen, Hungary*
[c] *3rd Department of Internal Medicine, University Medical School of Debrecen, P.O. Box 1, H-4012 Debrecen, Hungary*

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Abstract The single stranded [^{3}H]oligonucleotide uptake by HL-60 human promyelocyte and K562 human erythroleukemia cells was stimulated 20–45-fold by DUSF (DNA uptake stimulating protein), and this effect was drastically reduced (to 1.6–13×) if the cells were induced to differentiate. The oligonucleotide uptake stimulating effect of DUSF was not altered in HL-60 and K562 cells, if the proliferation of the cells was inhibited by hydroxyurea (HU) treatment. The oligonucleotide uptake by separated granulocytes and mononuclear cells from healthy donors was not stimulated by DUSF, while the uptake of oligonucleotides by myeloid and lymphoid leukemic cells was greatly stimulated (10–15×). The uptake of oligonucleotides by differentiated mononuclear cells of healthy donors could not be stimulated by DUSF, but the oligonucleotide uptake was greatly increased (11×) by DUSF if the cells were subjected to blast transformation.

Key words: Oligonucleotide uptake; DNA uptake stimulating protein; Leukemia; Cell proliferation; Antisense oligonucleotide

1. Introduction

It has been known since more than a decade that mammalian cells can take up DNA and oligonucleotides, although the molecular mechanism of uptake and its biological relevance have remained obscure [1–4]. The introduction of antisense oligonucleotides into mammalian cells is thought to be of major therapeutic importance in the future [5,6]. Antisense oligonucleotides may specifically inhibit virus propagation, division of tumor cells, or the expression undesirable genes by annealing to coding sequences of DNA or to mRNA [7,8]. A limitation of application of antisense oligonucleotides is based upon the low level of uptake by mammalian cells under physiological conditions. Therefore the stimulation of uptake would be desirable.

In a previous publication the occurrence of a natural DNA uptake stimulatory factor (DUSF), a protein molecule that appears in the culture medium of *Neurospora crassa* strain (FGSC 1118, slime) was reported [9]. DUSF was purified to homogeneity, biochemically characterized [10], and found to be a cytoplasmic membrane associated protein able to bind DNA and nucleotides [11]. The optimal conditions of DUSF action were described and its possible biological role in the uptake of DNA and oligonucleotides was discussed [11]. It was also described (i) that DUSF was able to stimulate oligonucleotide and linearized pBR322 plasmid DNA uptake into mammalian tumor cells in vitro; (ii) it has also been demonstrated that macromolecular DNA was not degraded during the uptake process; and (iii) DUSF antigen could also be detected in the cytoplasmic membrane of these malignant cells [12].

In this paper we demonstrate that DUSF does stimulate single stranded (ss) [^{3}H]oligonucleotide uptake by primitive leukemia cells, while it shows very limited activity with respect to differentiated forms.

2. Materials and methods

2.1. Cell cultures

HL-60 human promyelocytic, K562 human erythroleukemia, and human mononuclear cells were propagated in RPMI 1640 medium (Gibco, USA) supplemented either with 20% (HL-60) or with 10% (K562, mononuclear cells) fetal calf serum (SEBAK, Germany), in plastic petri dishes having a diameter of 9 cm (Greiner, Germany). The media were also supplemented with penicillin, streptomycin and/or gentamycin. All mammalian cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Preparation of DUSF and single stranded [^{3}H]oligonucleotides

The DNA-uptake-stimulating protein (DUSF) was isolated from the culture medium of *N. crassa* cell-wall-less mutant strain (FGSC 1118 [fz; sg; os-1, slime]) as described earlier [10]. The mixture of ss [^{3}H]oligonucleotides was produced by limited digestion of [^{3}H]DNA (extracted from *N. crassa* cells grown in the presence of [^{3}H]adenine) with bovine pancreas DNase I (Sigma) 25 μg/ml at 37°C, for 60 min. After termination of the enzyme treatment the [^{3}H]oligonucleotides were denatured at 100°C for 10 min and immediately chilled in a 0°C ice bath. The ss [^{3}H]oligonucleotides were separated from the reaction mixture by Sephadex G75 gel filtration and their polyacrylamide gel electrophoresis characterization revealed approximately 10–40-bp long fragments.

2.3. Uptake of single stranded [^{3}H]oligonucleotides

Cells were washed three times in Hank's buffer and then a suspension of 3×10^6 cells/ml in the same buffer was prepared. Incubation mixture consisted of 1 ml cell suspension, 0.02 ml DUSF (20 μg) or fresh medium and 0.01 ml of ss [^{3}H]oligonucleotides (10 μg). Incubation was carried out with shaking in a water bath at 37°C for 2 h. The uptake was terminated by rapid cooling of the samples for 10 min at 0°C. [^{3}H]Oligonucleotides not taken up by the cells were eliminated by the addition of 0.2 mg DNase I for 10 min at 37°C, and immediately

*Corresponding author. Fax: (36) (52) 416-531.

[1]Deceased. The author collective left behind dedicates this paper to the memory of Professor Gábor Szabó, M.D., Member of the Hungarian Academy of Sciences.

Abbreviations: Ara-C, cytosine-β-D-arabinofuranoside; B-CLL, B-cell chronic lymphoid leukemia; CGL, chronic granulocytic leukemia; DNase I, deoxyribonuclease I (EC 3.1.21.1); DUSF, DNA uptake stimulating factor; HU, hydroxyurea; PHA, phytohemagglutinin; ss, single stranded; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; WBC, white blood cell

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Table 1
The effect of DUSF on the uptake of ss [^{3}H]oligonucleotides by proliferating undifferentiated and differentiated mammalian cells

Mammalian cells	Uptake of ss [^{3}H]oligonucleotides (dpm/mg dried cell ± S.D.)					
	Proliferating undifferentiated cells			Differentiated cells		
	−DUSF	+DUSF	Stimulation	−DUSF	+DUSF	Stimulation
HL-60	105 ± 15	2106 ± 156	20.0×	287 ± 50	448 ± 65	1.6×
K562	223 ± 36	10 115 ± 280	45.4×	276 ± 20	3640 ± 173	13.2×

In vitro proliferating undifferentiated tumor cells were differentiated by the addition of TPA (HL-60 cells) or Ara-C (K562 cells), respectively, as described in Section 2. The uptake of [^{3}H]oligonucleotides (10 μg/ml) was measured in the absence (−) and in the presence (+) of DUSF (20 μg/ml). Data represent the mean ± S.D. of three separate determinations.

washed off in four repeats of 0.5 M NaCl at 0°C. Finally the cells were dried in the centrifuge tubes. The radioactivity of the samples was measured as in [11]. Data shown in the tables are the mean values ± S.D. of three separate determinations.

2.4. Human granulocyte and mononuclear cell suspensions

Granulocytes were separated from heparinized venous blood samples obtained from voluntary healthy donors and patients with chronic granulocytic leukemia (CGL), in the blast phase of their disease [13]. These human white blood cell (WBC) suspensions used in the experiments consisted of 95% granulocytes and 5% mononuclears but no red blood cells.

Mononuclears were separated from heparinized venous blood of voluntary healthy donors and patients with B-cell chronic lymphoid leukemia (B-CLL) [14]. The final mononuclear cell suspension of healthy donors consisted of 70–80% lymphocytes and 20–30% monocytes, while that of the B-CLL patients contained 95% lymphoid cells (the majority of them being morphologically differentiated) and 5% monocytes. Trypan blue exclusion test was performed to determine the viable cell fraction [15], which demonstrated 90–95% viability.

2.5. Differentiation of HL-60 and K562 cells in vitro

HL-60 human promyelocytic leukemia cells which grow in suspension in vitro are ready to undergo differentiation into macrophage-like cells [16]. 72 h after the addition of *O*-tetradecanoylphorbol-13-acetate (TPA, Sigma, final concentration 300 nM) to the medium nearly 100% of the cells became adherent to the bottom of the tissue culture dish and exhibited positive alpha-naphthylacetate esterase reaction as signs of differentiation [17]. Cells adhering to the plastic surface were scraped off by a rubber policeman and resuspended in fresh medium. 85–90% of the cells proved to be viable by the trypan blue dye exclusion test.

K562 human erythroleukemia cells were differentiated towards the erythroid route by addition of cytosine-β-D-arabinofuranoside (Ara-C, Sigma, final concentration 10 μM) to the culture medium [18]. 72 h later the cells were collected by centrifugation (500 rpm, 5 min, 37°C) and resuspended in fresh medium. The differentiation of the cells and its degree was estimated by the detection of embryonic and fetal hemoglobins within the cells by means of the benzidine reaction [18]. 68% of the Ara-C treated cells became benzidine positive while in the untreated control cultures their proportion was only 10%. 85% of the treated cells proved to be viable by the trypan blue dye exclusion test.

2.6. Blast transformation of lymphocytes

From heparinized venous blood of a healthy donor mononuclear cells were separated [14]. The cells were washed three times in RPMI 1640 medium containing 10% fetal calf serum, and resuspended in the same medium at a cell density of 10^6 cells/ml. The suspension was divided into two equal portions and cultivated in the presence or absence (control) of phytohemagglutinin (PHA, Sigma, final concentration 0.2 μg/ml) for 72 h as described above. Cell proliferation was followed by measuring [^{3}H]thymidine incorporation. PHA-treated cells after 72 h in culture incorporated 5 times more [^{3}H]thymidine than the controls. 90% of the cells in the parallel cultures proved to be viable by the trypan blue dye exclusion test.

3. Results and discussion

It was described previously [12] that DUSF stimulated oligonucleotide uptake by proliferating human HL-60 promyelocytic and K562 erythroleukemic cells in vitro. It remained intriguing, however, to see whether DUSF was able to stimulate oligonucleotide uptake of these cells in their differentiated state as well. HL-60 cells were switched to differentiate towards the macrophage route by TPA, while K562 cells were induced in the direction of erythroid development by Ara-C, and their oligonucleotide uptake was measured in the presence and absence of DUSF.

It is shown in Table 1 that the ss [^{3}H]oligonucleotide uptake stimulating effect of DUSF almost completely disappeared in the case of differentiated HL-60 cells, and with K562 differentiated cells a merely 1/3 stimulation could be seen compared to the proliferating undifferentiated cells. This lower degree of uptake, however, remained still comparable with that of the differentiated HL-60 cells. Two factors may be considered in the explanation of that difference: (i) both the basal and the DUSF-stimulated oligonucleotide uptake of the K562 cells are considerably higher, several times more than that of the proliferating HL-60 cells; and (ii) the degree of differentiation in the HL-60 culture was almost 100% as determined by cytochemical reaction, while the K562 population seemed to be more heterogeneous in this aspect showing signs of differentiation only in 68% of the Ara-C treated cells (see Section 2). It seems very likely that the oligonucleotide uptake stimulatory effect of DUSF decreases or even ceases with the tran-

Table 2
Proliferation (A) and ss [^{3}H]oligonucleotide uptake (B) in hydroxyurea-treated HL-60 cells in the presence and absence of DUSF

A	Untreated		Hydroxyurea-treated	
	0 h	24 h	0 h	24 h
Cell number (×10^5/ml)	0.77	2.31	0.77	1.19
Increase of cell number (%)	100	300	100	155
Trypan blue positivity (%)	10	12	10	13

B	Uptake of ss [^{3}H]oligonucleotides (dpm/mg dried cell ± S.D.)	
	Untreated	Hydroxyurea-treated
−DUSF	120 ± 20	100 ± 30
+DUSF	2663 ± 135	2846 ± 170

Suspensions of HL-60 cells proliferating in vitro were split and to one half of the cultures HU was added at a final concentration of 0.2 mM. The other halves served as controls. The cells were further cultivated for another 24 h. The viability of the cells was estimated by the trypan blue exclusion test [14]. The [^{3}H]oligonucleotide uptake was determined in the absence (−) and in the presence (+) of DUSF (20 μg/ml) as described in Section 2. Data represent the mean ± S.D. of three separate determinations.

Table 3
Proliferation (A) and ss [^{3}H]oligonucleotide uptake (B) in hydroxyurea-treated K562 cells in the presence and absence of DUSF

A	Untreated		Hydroxyurea-treated	
	0 h	24 h	0 h	24 h
Cell number ($\times 10^5$/ml)	0.82	2.07	0.82	1.01
Increase of cell number (%)	100	252	100	123
Trypan blue positivity (%)	10	12	12	14

B	Uptake of ss [^{3}H]oligonucleotides (dpm/mg dried cell ± S.D.)	
	Untreated	Hydroxyurea-treated
−DUSF	110 ± 20	140 ± 30
+DUSF	4736 ± 174	4530 ± 298

Suspensions of K562 cells proliferating in vitro were split and to one half of the cultures HU was added at a final concentration of 5 mM. The other halves served as controls. The cells were further cultivated for another 24 h. The viability of the cells was estimated by the trypan blue exclusion test [14]. The [^{3}H]oligonucleotide uptake was determined in the absence (−) and in the presence (+) of DUSF (20 μg/ml) as described in Section 2. Data represent the mean ± S.D. of three separate determinations.

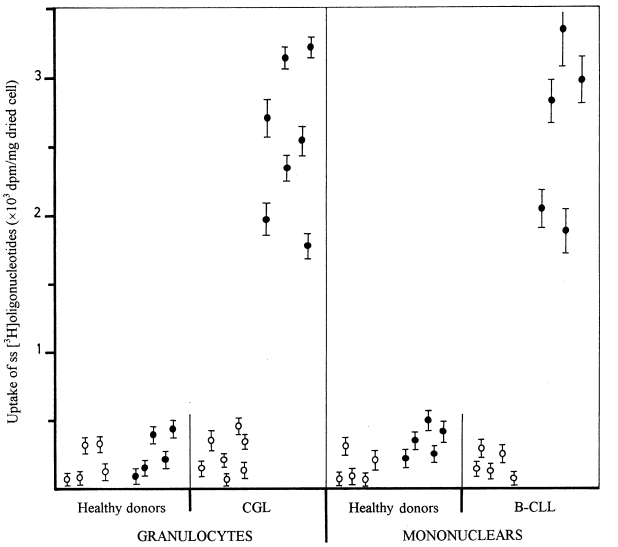

Fig. 1. The effect of DUSF on the uptake of ss [^{3}H]oligonucleotides by human granulocytes and mononuclears of healthy donors or leukemic patients. Granulocyte and mononuclear cell suspensions were prepared from venous blood of healthy donors (controls) as well as from patients with chronic granulocytic leukemia (CGL) or B-cell chronic lymphoid leukemia (B-CLL), as described in Section 2. The uptake of [^{3}H]oligonucleotides (10 μg/ml) by these cells was determined in the absence (○) and in the presence (●) of DUSF (20 μg/ml). Each symbol represents the mean of three determinations (± S.D.). Cell samples of donors were halved and determinations of [^{3}H]oligonucleotide uptake were made on the same populations regarding the presence and absence of DUSF (see Section 2).

sition of the tumor cells from the primitive proliferating to the differentiated stage.

It is well known that malignant cells divide frequently, and their differentiation is accompanied by a decrease in the rate of proliferation. Thus it proved itself a challenging problem to establish whether the decrease of the oligonucleotide stimulating ability of DUSF in the case of TPA and Ara-C treated leukemic cells could be considered as a consequence of differentiation of those tumor cells, or it was connected to the cessation of cell proliferation.

In order to address that question we studied the ss [^{3}H]oligonucleotide uptake in the normally proliferating state of the tumor cells as well as when their propagation was inhibited. HL-60 and K562 cells were cultivated either in the presence or in the absence of hydoxyurea (HU) for 20 h prior to the determination of oligonucleotide uptake with and without the DUSF. (HU is a potent inhibitor of the enzyme ribonucleotide reductase and thus of DNA synthesis, and it halts progression through the cell cycle evoking cessation at the G1/S boundary [19].)

Parts 'A' in Table 2 and in Table 3 as well, show a marked decrease in the proliferation of HL-60 and K562 cells, respectively, without diminishing the viability of the cells. The trypan blue exclusion test did not show differences between the HU-treated and non-treated cells. In parts 'B' of Tables 2 and 3, respectively, we demonstrate that the basal and the DUSF-stimulated [^{3}H]oligonucleotide uptake was essentially the same in the HU-treated, in their propagation blocked tumor cells and in the non-treated, proliferating cultures.

These results may suggest us that the decrease of the stimulating effect of DUSF is rather the consequence of the differentiation of the tumor cells than it would be the result of the inhibition of cell proliferation. It has to be noted, however, that differentiation of tumor cells is accompanied by their lower rate of proliferation.

From a practical point of view it seemed to be of utmost importance to compare the effect of DUSF upon oligonucleotide uptake by normal and tumor cells of human origin. To address this question granulocytic and lymphoid cells were separated from the blood of patients either with blast phase CGL or B-CLL, respectively. Normal granulocytes and mononuclear cells were obtained from peripheral blood of healthy donors. The uptake of ss [^{3}H]oligonucleotides by these cell populations was measured in the presence and absence of DUSF.

It is shown in Fig. 1 that DUSF failed to stimulate oligonucleotide uptake by normal granulocytes and lymphocytes but the uptake was considerably enhanced in the case of leu-

Table 4
The effect of DUSF on ss [^{3}H]oligonucleotide uptake in control and in PHA-treated mononuclear cell populations

Mononuclear cells	Uptake of ss [^{3}H]oligonucleotides (dpm/10^6 cells)		
	−DUSF	+DUSF	Stimulation
Non-treated	211 ± 27	372 ± 32	1.7×
PHA-treated	428 ± 15	4606 ± 142	10.8×

Mononuclear cells were separated from venous blood of healthy donors. Half of the cell suspension was treated by phytohemagglutinin (PHA, 0.2 μg/ml) as described in Section 2. The uptake of [^{3}H]oligonucleotides (10 μg/ml) by these cells was determined in the absence (−) and in the presence (+) of DUSF (20 μg/ml). Data represent the mean ± S.D. of three separate determinations.

kocytes from CGL and B-CLL patients in the blast phase. This apparent difference might be due to the fact that there are ultimately differentiated WBCs in the circulation of healthy individuals, while some of the WBCs in the peripheral blood of malignant myeloid or lymphoid leukemia patients are undifferentiated progenitor cells [20–22]. In our experiments smears of cells isolated from patients' blood samples revealed more than 35% blast phase primitive cells in cytological analysis.

Our observations are in good agreement with results of other authors who (i) reported that oligonucleotide uptake was stage-specific during B cell differentiation, namely, an enhanced oligonucleotide uptake ability in B progenitor cells in bone marrow was detected as compared to low values experienced in mature B cells [23]; (ii) described enhanced oligonucleotide uptake by in vitro proliferating tumor cells isolated originally from acute myeloid or lymphoid leukemia patients, compared to healthy counterparts [24].

Based on the results shown in Table 1 and in Fig. 1 it was assumed that DUSF was unable – or had a very limited ability – to stimulate oligonucleotide uptake by differentiated cells, and it was predominantly active with cells progressing through the cell cycle. This hypothesis was tested in the following experiment.

Mononuclear cells were separated from venous blood of healthy donors. The cell suspensions were split into two equal parts, one being induced by the mitogen PHA and the other not (control). Each of the suspensions were further split into two equal parts, and the ss [^{3}H]oligonucleotide uptake was determined in the presence and absence of DUSF. Table 4 shows that the oligonucleotide uptake stimulating effect of DUSF was almost negligible in the case of differentiated cells while it was substantial in the mitogen induced blast transformation population.

Previous reports by others have already described enhanced oligonucleotide uptake by mitogen (concanavalin A or lipopolysaccharide) stimulated T and B lymphocytes [25,26]. It was recently published by Zhao et al. [24] that proliferating bone marrow cells stimulated by hematopoietic growth factors (interleukin-3, stem cell factor) showed considerably enhanced oligonucleotide uptake on the one hand, and on the other, in HL-60 cells the oligonucleotide uptake was decreased by the induction of cell differentiation with all-*trans*-retinoic acid.

The results cited as well as our observations are suggestive of a possible correlation between the oligonucleotide uptake ability of mammalian cells and their activated and/or differentiated stage.

The oligonucleotide uptake stimulating potential of DUSF seems to us to be predominantly effective in undifferentiated in vitro proliferating leukemic cells (Table 1), in malignant tumor cells (Fig. 1), and in mitogen blast transformed lymphocytes (Table 2), while this protein remained practically ineffective in differentiated cell populations (cells induced to differentiate in vitro, normal WBCs). The ability of cells to take up oligonucleotides from their environment seems to be dissimilar in the differentiated and in the primitive proliferating state. It has not been possible to determine the real cause of this phenomenon yet, on the one hand, and on the other it is rather plausible to assume the expression of proteins involved in oligonucleotide uptake being higher in primitive, undifferentiated state of tumor cells.

Several proteins have already been detected on the surface of a number of various eukaryotic cells which are able to bind nucleic acids, but their eventual role in the uptake of nucleic acids has not been proved yet [27–29]. To our recent knowledge DUSF is the only isolated protein which is able to enhance the uptake of oligonucleotides (and macromolecular DNA) into mammalian cells. DUSF might act for instance as a receptor protein, since (i) DUSF proved to be capable to bind DNA and nucleotides as well, and these molecules show a competition in that binding reaction, (ii) the presence of DUSF has been demonstrated on the surface of mammalian cells including tumor cells as well, and (iii) anti-DUSF antibodies have been shown to be capable to inhibit basal- and DUSF-stimulated uptake of DNA/oligonucleotides in tumor cells [11,12].

The fact that DUSF is capable to increase the uptake of single stranded oligonucleotide(s) by primitive leukemic cells, may give some good hope of an eventual promotive effect in the uptake of proliferation inhibiting antisense oligonucleotides as well.

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