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Review

Advances in magnetofection—magnetically guided nucleic acid delivery

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Abstract

Magnetofection is nucleic acid delivery to cells supported and site-specifically guided by the attractive forces of magnetic fields acting on nucleic acid shuttles (vectors) which are associated with magnetic nanoparticles. Recent progress with the method confirms its general applicability with small and large nucleic acids and viruses. The method's therapeutic application as well as mechanistic studies will be discussed.
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1. Introduction

Nucleic acids are the carriers of the building plans of living systems, are involved in most aspects of cellular information encoding and transmission and as such contribute directly and indirectly to the coordination and regulation of cellular processes. This distinguished role of nucleic acids has led to the idea that any process within living cells, in theory, can be purposefully influenced by the introduction of nucleic acids into living cells from without. This strategy has been explored during the last forty years and has led to nucleic acid delivery protocols which are now powerful research tools and which emerge as potent therapeutic modalities (gene therapy). The

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objectives of nucleic acid delivery in research and therapy are the overexpression of a particular gene, or the silencing or knock-down of a selected gene or the actual correction of genetic defects by DNA repair mediated by transfected nucleic acid molecules. Depending on the actual purpose, larger or smaller nucleic acid constructs need to be introduced into cells. Larger constructs include genes inserted in plasmid DNAs, artificial chromosomes or viral genomes, examples of smaller constructs are synthetic oligonucleotides (antisense, small interfering RNAs) or messenger RNAs. The challenge of nucleic acid delivery into cells has been solved by nature itself in the form of viruses. Not surprisingly, genetically modified viruses (viral vectors) are the most efficient shuttles that are currently used for introducing nucleic acids into cells. For various reasons, including the costs of manufacturing, aspects of practicability and the potential of biological risks, researchers have focused on constructing and optimizing synthetic alternatives to viruses as nucleic acid shuttles.

No matter whether large or small nucleic acid constructs need to be transferred, whether viruses or synthetic vectors are used for delivery, the delivery process itself is a diffusion limited one. In other words, the time that is required until vectors encounter their target cells by simple diffusion and bind to their surface constitutes a major limitation for successful nucleic acid delivery [1]. This limitation is even more pronounced in vivo (living animals or patients) where opsonization, the immune system and degradative processes inactivate vectors and where vectors can be purged from the target tissue by the blood stream even upon local administration. Particularly in vivo, the local confinement of nucleic acid delivery is an important requirement in many applications. First of all, local targeting may be required to achieve an effective dose at a target site and secondly, local confinement is an objective in order to reduce side-effects at non-target sites [2].

In this background, we have adapted the principles of magnetic drug targeting to nucleic acid delivery. Here, we demonstrate ways of non-covalently associating vectors, viral and non-viral and large and small nucleic acids with polyelectrolyte-coated iron oxide particles, show that these magnetic vectors can be targeted by magnetic fields in vitro and in vivo and that magnetic guidance greatly improves both the efficacy and the kinetics of nucleic acid and gene delivery [2,3].

2. Generation of magnetic vectors

Physical, chemical and biological linkages can be envisaged for associating nucleic acids or nucleic acid vectors to magnetic nanoparticles. Depending on the linkage type chosen, magnetic nanoparticles have to be provided with a suitable surface coating. In our studies we have chosen physical linkage while other researchers used biotin–streptavidin linkages [4–7]. We used magnetite particles with a hydrodynamic diameter of 50, 100 or 200 nm with a saturation magnetization of $J = 5.5\,\mathrm{mT}$ (chemicell, Berlin, Germany). These particles are coated with polyelectrolytes such as cationic or anionic poly(amino acids), polyethylenimine (PEI), phosphorylated starch, DEAE dextran or similar compounds. These charged colloids are subject to salt-induced aggregation/flocculation, a phenomenon well known in colloid science [8]. Nucleic acid vectors are charged nanoparticles as well, synthetic virus-like vectors usually are positively charged. Therefore, the simplest way of associating magnetic nanoparticles with vectors is to mix polyelectrolyte-coated magnetic particles with the vectors in salt-containing buffer [9]. In this manner, positively as well as negatively charged magnetic particles will associate with positively charged vectors. Viral vectors usually display negative surface charge and display size stability at physiological salt concentration. These vectors associate with positively charged magnetic particles by electrostatic interaction. Therefore, our primary choice in magnetically guided nucleic acid delivery were polycation-coated magnetic particles, particularly PEI-coated ones as these are generally applicable to naked nucleic acids, synthetic vectors and viral vectors. It needs to be emphasized though, that negatively charged magnetic particles are equally suited for non-viral vectors [9].

The preferred choice of PEI-coated particles was based on the fact that this molecule is known as an excellent synthetic transfection reagent itself and is actually one of the most widely used compounds in synthetic gene delivery systems. Associated with nucleic acids, it forms nanoparticles that bind to cells via unspecific electrostatic interactions, and which are internalized by the natural process of endocytosis. The chemical structure of PEI implies buffering capacity at physiological pH which contributes to the release of the nucleic acid from endosomes into the cytoplasm (the so-called proton sponge effect [10]). Coating of magnetic nanoparticles with PEI confers to them not only nucleic acid and vector binding capacity but also the potential to exert the proton sponge effect once internalized into cells. Optimized versions of PEI-coated magnetic particles available from chemicell, Berlin, Germany and OZ Biosciences, Marseille, France, are provided with a multilayer PEI coating which by itself promotes efficient intracellular processing of internalized vectors. It is an important characteristic of the described magnetic vectors that the linkage between magnetic particles and nucleic acid/vector is reversible. In this manner, the intracellular steps which are required for functional nucleic acid delivery are not compromised.

3. Magnetofection—magnetically guided nucleic acid delivery

The fundamental principle of magnetofection is simple and comprises the steps of formulating a magnetic vector as described above, adding it to the medium covering cultured cells or injecting it systemically via the blood stream or applying it locally to a target tissue, and in addition applying a magnetic field in order to direct the vector towards the target cells or retain it in the target tissue, respectively. Fig. 1 shows the principle of magnetofection in cell culture. For these experiments, we have developed magnetic plates upon which the culture plates are positioned during magnetofection [3]. In this manner, the magnetic vectors which originally are dispersed in the culture supernatant are sedimented within minutes on the target cells, breaking the diffusion barrier to nucleic acid delivery that has been mentioned above. The consequence of this is that virtually the whole applied vector dose gets in contact with the cells to be transfected contributing to an increase in the efficiency of the process up to several orders of magnitude. In addition, the time required for a transfection process is greatly reduced. Favourable kinetics and exploiting the full applied vector dose in turn lead to a highly favourable dose–response relationship in comparison with non-magnetic standard vectors: a lower dose and extremely short incubation times yield the same or superior efficiencies than higher doses of standard vectors at longer incubation times [2,9]. This is particularly important if the presence of the vector cocktail in the culture medium over extended periods of time provokes adverse effects in the cells to be transfected [11].

It has been mentioned above that suitably coated magnetic nanoparticles can be associated virtually with any non-viral and possibly also with any viral vector construct by the mentioned physical interactions. In our previous studies we have demonstrated that in fact the efficiencies of all the non-viral vectors we have "magnetofected" so far and of adenoviral and retroviral vectors could be improved. Magnetofection with adenoviral vectors even had the consequence that these vectors then were able to infect cells that otherwise are non-permissive to infection with this virus [3]. The versatility and simplicity of the method allows the user to easily adapt the system to his/her favourite nucleic acid vector. Several groups have used commercially available streptavidin-coated magnetic particles to link these with biotinylated viruses [4–7] and have observed similarly encouraging results as we did.

Our original report on magnetically guided nucleic acid delivery gave rise to the speculation that the magnetic field itself might have an impact on nucleic acid transfection by an unknown influence on cell physiology. Our early results, however, had already indicated that the actual uptake mechanisms of vectors into cells remain unchanged [3]. The studies by Huth et al. confirm this interpretation at least for synthetic PEI-based magnetic vectors [12]. Transfection experiments in

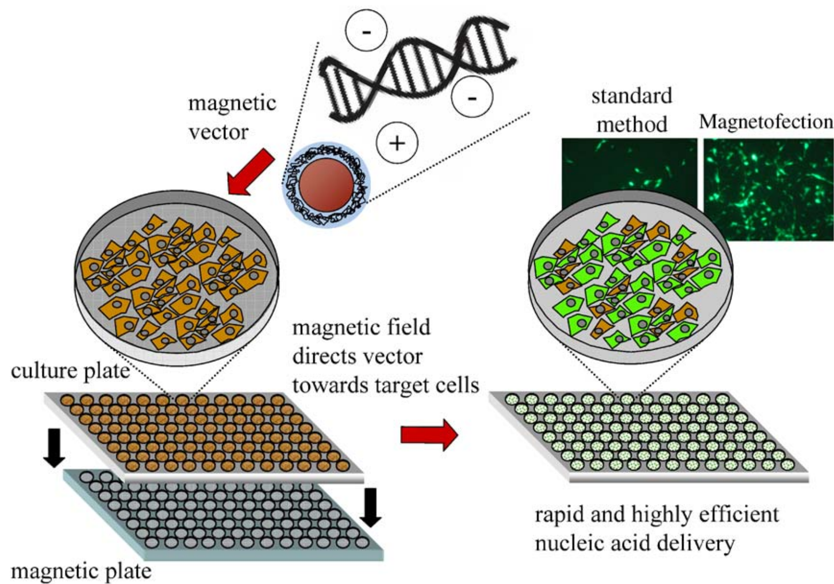

Fig. 1. Principle of magnetofection in cell culture. Polyelectrolyte-coated magnetic nanoparticles are mixed with naked nucleic acids or synthetic or viral nucleic acid vectors in salt-containing buffer. The particles associate with nucleic acids and vectors by electrostatic interaction and/or salt-induced colloid aggregation. The mixtures are added to cells in culture. The cell culture plate is positioned on a magnetic plate during 5–30 min of incubation. The magnetic field(s) rapidly sediment vectors on the cells to be transfected/transduced. The result is rapid kinetics and high efficiency nucleic acid delivery. Shown is a cell culture plate and a magnetic plate in 96-well format. The magnetic plate consists of 96 individual neodymium–iron–boron magnets (IBS Magnets, Berlin, Germany) inserted in drill holes in an acrylic glass or PVC plate in strictly alternating polarization. The plate was designed for application with 96-well cell culture plates but is also applicable for 24-, 12- and 6-well layouts, petri dishes of various diameters and culture flasks of various sizes. Detailed protocols can be found at www.ozbiosciences.com.

the presence of various agents that inhibit or influence specific steps during endocytosis showed that the cellular uptake of these constructs proceeds via clathrin-dependent and caveolae-dependent endocytosis apart from unspecific macropinocytosis. These conclusions were further supported by electron microscopy which demonstrated that magnetic particles and the nucleic acid are co-internalized into cells (Fig. 2). The function of the magnetic field in magnetofection appears to be limited to what it was supposed to be, namely to concentrate magnetic vectors on the target cells.

4. Magnetofection is applicable in primary cells with small and large nucleic acids and mediates a therapeutic effect in feline fibrosarcoma

The introduction of nucleic acids into cells is relevant for therapeutic (gene therapy) purposes only if it is feasible for primary cells, e.g. non-immortalized cells freshly isolated from an organism (a patient). We confirmed the potency of the magnetofection method with primary cells including lung epithelial cells [13], blood vessel endothelial cells [14], keratinocytes, chondrocytes (Fig. 3), osteoblasts and amniocytes (unpublished results) as well as with whole tissue specimens of airways [13] and with blood vessels ([3,11] and unpublished results). With the latter we demonstrated an extremely efficient and rapid delivery of synthetic oligonucleotides used in antisense strategies to specifically shut down the expression of selected genes [11]. In this respect, we established the method as a powerful tool in molecular physiological research and at the same time demonstrated its potential in nucleic acid-based therapies of cardiovascular diseases. As part of this work, we also showed the potency of magnetofection for the delivery of siRNA (small interfering RNA), an

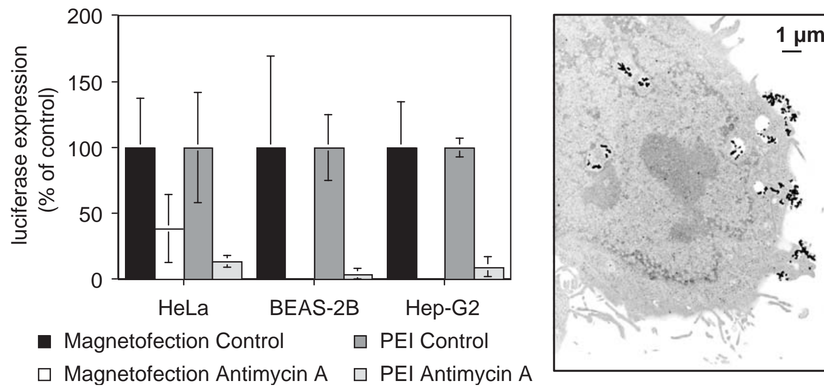

Fig. 2. Huth et al. have examined the mechanism of magnetofection by performing gene delivery experiments in the presence of various inhibitors of endocytosis [12]. Left: HeLa (human cervix carcinoma), BEAS-2B (bronchial epithelial) and Hep-G2 (human hepatocellular carcinoma) cells were seeded at 10,000 cells/well in 96-well plates the day prior to transfection. Standard transfection was carried out with PEI-DNA complexes (N/P ratio = 6) with 250 ng DNA/well, magnetofection was carried out with 500 ng DNA/well using transMAG-PEI magnetic nanoparticles (chemicell, Berlin, Germany) mixed with plasmid DNA coding for firefly luciferase. Dark bars: Control experiments in the absence of endocytosis inhibitors; light bars: Transfections in the presence of antimycin A (final concentration of 1 μg/ml) during transfection. This agent unspecifically blocks endocytosis [20]. The luciferase reporter gene assay was carried out 24 h post transfection. Further details have been published by Huth et al. The results demonstrate that an unspecific inhibitor of endocytosis efficiently blocks gene delivery both by the standard method (PEI-DNA) and by magnetofection. Huth et al. have demonstrated that similar results are also obtained with other, more specific inhibitors of endocytosis and intracellular transport. Right: Electron micrograph of HeLa cells 24 h after magnetofection carried out similarly as described above in the absence of antimycin A. Magnetic particle–DNA complexes are found at the cell surface and in intracellular structures that often appear to be membrane-surrounded. For more details see Huth et al. [12].

exciting novel method of gene knock-down [2]. Fig. 4 shows the knock-down of luciferase reporter gene expression in a cell line (HeLa) that had previously been stably transduced with the reporter gene using a retroviral vector.

An efficient nucleic acid delivery technology like magnetofection is certainly useful for research applications. It remains to be demonstrated that it is also suitable for therapeutic applications. Most current efforts in gene therapy aim at cancer. Among the many strategies that have been pursued, immuno gene therapy has been one of the most frequent approaches. One such strategy is introducing cytokine genes into tumor cells ex vivo which, when re-administered after irradiation to a patient, are supposed to elicit an immune response against tumor antigens due to the immunostimulatory effect of the expressed cytokine (for a recent review of cancer immuno therapy strategies, in general, see Ref. [15]). The immune response is hoped to be sufficient to eliminate residual tumor cells which could not be removed by surgery, which in most cases is the primary therapeutic intervention. Another strategy is the direct injection of cytokine-encoding gene vectors into tumors, essentially pursuing similar goals as just mentioned [16]. One frequently used cytokine gene is the one coding for granulocyte macrophage colony stimulating factor (GM-CSF). This cytokine supports the recruitment of antigen-presenting cells upon intratumoral injection of its gene [17]. We want to exploit this activity in conjunction with magnetofection as the gene delivery system for direct intratumoral injection. In this case, the magnetic guidance is not supposed to direct the vector towards its target cells but rather is supposed to hold the injected dose within the tumor. Usually, artificial mouse tumor models are exploited to demonstrate the potentials of novel therapeutic modalities. The advantage of such models is that they are well established and that details, of an immune response, for example, can be examined. The disadvantage is that the models are artificial and often are not predictive of what

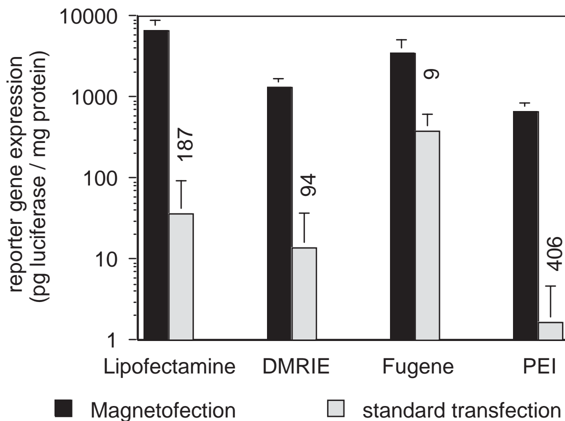

Fig. 3. Comparison of magnetofection and standard transfection in primary rabbit articular chondrocytes (passage 2). Cells were plated and transfected in a 96-well plate. DNA complexes were prepared by either adding DNA (one volume equivalent; 10 μg/ml in DMEM) to the transfection reagent (one volume equivalent) or by first mixing one volume equivalent of DNA (20 μg/ml in DMEM) with one volume equivalent magnetic particles (transMAG-PEI; 20 μg/ml; chemicell, Berlin, Germany) followed by addition to two volume equivalents of transfection reagent diluted in DMEM (Lipfectamine: 4 μl per μg of DNA; DMRIE: 5 μl/μg DNA; Fugene: 3 μl/μg DNA; PEI: 1.042 μg/μg DNA). Lipofectamine and DMRIE reagents were purchased from Invitrogen, Fugene from Roche, and branched PEI 25 kD from Sigma-Aldrich. The cell culture plate was positioned on the magnetic plate for 15 min. Determination of luciferase expression was carried out after 24 h. Bars show averages and standard deviations from triplicates. Numbers above the grey bars indicate—fold enhancements by magnetofection.

would happen in the real world in spontaneous tumors [18]. Therefore, we initiated a veterinary clinical study of immuno gene therapy of feline fibrosarcoma after having demonstrated that direct intratumoral injection of a magnetic particle–DNA vector results in the site-specific transfection of a tumor. For this purpose, a hamster which had developed a spontaneous, probably age-related breast tumor was injected with 200 μg plasmid DNA coding for the luciferase reporter gene complexed with 200 μg of magnetic particles (transMAG-PEI, chemicell, Berlin, Germany) in a total volume of 500 μl isotonic saline directly into the tumor. A neodymium-iron-boron permanent magnet ($2 \times 1 \times 0.5$ cm, Neodelta, IBS Magnet, Berlin, Germany) was fixed on the tumor adjacent to the injection site during one hour after vector injection. Forty hours later, the animal was sacrificed and the tumor and various organs (heart, lung, liver, spleen, skin above the tumor) were assayed for luciferase expression. Only in the tumor, this sensitive assay detected expression of the transfected gene (17 pg luciferase per gram tissue) and not in any of the other examined organs. This observation was confirmed in other experiments involving magnetofection by the same way of administration in tumor-bearing mice (data not shown). Together with other previous results from our group [3,13], this confirmed that local gene delivery can be achieved by magnetofection, setting the basis for initiating a veterinary clinical study.

Feline fibrosarcoma is one of the most common feline tumors with a relapse rate of 75% within 6 months upon surgical resection, which is the standard therapy (for more details on feline fibrosarcoma, see Ref. [19]). The gene coding for human GM-CSF (granulocyte macrophage colony stimulating factor) under the control of the CMV promoter in magnetic formulation was administered twice in a 1 week interval prior to surgery into the biologically active margins of the fibrosarcoma. The dose was 1.25 mg of plasmid DNA mixed with 1.25 mg of magnetic particles (transMAG-PEI, chemicell, Berlin, Germany) in a total volume of 500 μl physiological saline. A neodymium–iron–boron permanent magnet ($2 \times 1 \times 0.5$ cm, Neodelta, IBS Magnet, Berlin, Germany) was fixed on the tumor adjacent to the injection site during one hour after vector injection. Expression of the magnetofected cytokine gene in the tumor was demonstrated by immunohistochemistry (Fig. 5). The application was well tolerated by the cats and a phase I study showed no adverse events (examined with a modified NCI GC-catalogue). The preliminary clinical outcome after a phase II study with more than 20 patients is a significant increase in tumor-free survival of the cats from only 23% at the 1 year time point in the case of standard therapy (surgery only) to 52% with pre-surgical magnetofection of the human GM-CSF gene. Additional patients have been admitted to the magnetofection group such that long-term follow-up will warrant a profound assessment of the benefits of this treatment.

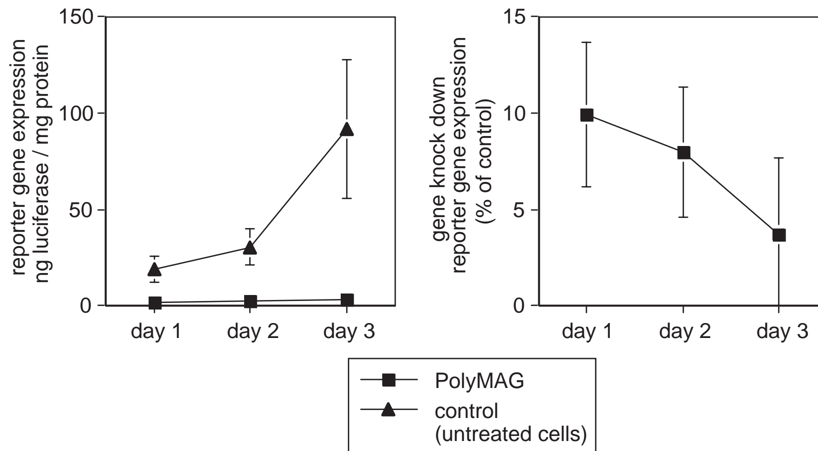

Fig. 4. Gene knockdown using magnetofection of siRNA (small interfering RNA). HeLa cells were transduced with a MLV-based retrovirus coding for luciferase as a reporter gene. This results in a population of cells stably expressing the reporter gene. These cells were seeded in a 96-well plate at a density of 7000 cells/well the day prior to siRNA transfection. Synthetic siRNA with the target sequence 5′-CTT ACG CTG AGT ACT TCG A-3′ was purchased from MWG-Biotech, Ebersberg, Germany. Stock solutions (2.5 μg/ml, corresponding to ca. 151.2 nM in DMEM) were mixed with the reagent PolyMAG available from OZ Biosciences, Marseille, France or chemicell, Berlin, Germany at a ratio of 1 μl PolyMAG per μg siRNA. Aliquots of 50 μl were added to the cells which were covered with 150 μl medium (DMEM, 10% fetal calf serum), resulting in a final siRNA concentration of 37.8 nM. The culture plate was positioned on a 96-well magnetic plate for 15 min. Luciferase expression was determined 24, 48 and 72 h post-transfection.

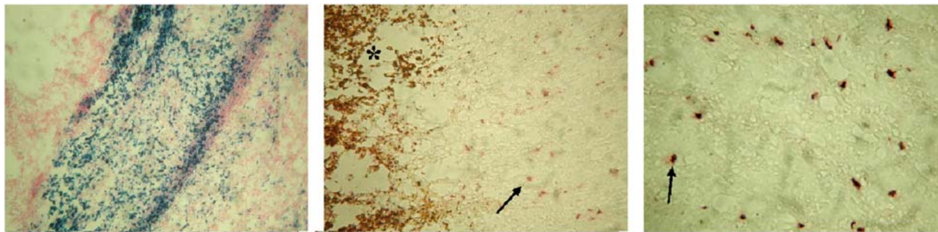

Fig. 5. Immunohistochemical staining for hGM-CSF expression in magnetofected and surgically removed tumors. Serial sections (8 μm) of cryo-embedded tumors were prepared and mounted on slides. The tissue samples were treated with blocking solution (10% fetal calf serum/0.05% Tween 20 in phosphate buffered saline) for 20 min, followed by incubation with a primary monoclonal anti-human GM-CSF antibody (R&D Systems, Wiesbaden, German. # MAB215; dilution 1:200) in blocking solution overnight at 4 °C. The samples were washed three times with PBS/0.05% Tween 20 and were then incubated for 1 h with a biotinylated secondary antibody directed against the primary antibody diluted in blocking solution (Anti-mouse IgG Biotin, Acris Antibodies, Hiddenhausen, Germany; # R1403B; dilution 1:500). The samples were washed again three times like above and were then incubated with ABC reagent (ABC Kit, Vector Laboratories, Burlingame, CA, USA; # PK 6200), followed by incubation with Vector VIP Peroxidase Substrate Kit (# SK 4600), both steps carried out according to the instructions of the manufacturer. After washing three times in distilled water, sections were covered with Kaiser's Gelatin (Merck, Darmstadt, Germany; # 1.09242.0100). Control stainings in neighbouring sections where any one of the components listed above was omitted showed no positive results (not shown). Other sections were stained using the Prussian blue staining method for the detection of magnetic particles which are also clearly visible as brownish agglomerates in immunohistochemical stains.

Left: Prussian blue staining shows retention of magnetic particles along the needle track (10 × magnification). Middle: immunohistochemical staining shows hGM-CSF expression in the vicinity of magnetic particles demonstrating some tissue penetration by the vector (100 × magnification). Right: Higher magnification (400×) of an immuno-stained section. Asterisk: magnetic particles in immuno-stained section. Arrows: cells staining positive for hGM-CSF expression.

5. Conclusions

Magnetically guided nucleic acid delivery can be practiced with viral and synthetic nucleic acid vectors, and can be used to overexpress nucleic acids or to silence endogenous gene expression. It can improve the efficacy of nucleic acid delivery by concentrating and/or retaining an applied vector dose at/in a target tissue both in primary cells in culture as well as in explanted tissue specimens and in living animals. Magnetically labeled non-viral vectors can exert a therapeutic effect when directly injected into naturally occurring tumors. In summary, these results establish and confirm magnetically guided nucleic acid delivery as a powerful tool in research and therapy.

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