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Internal ribosome entry sites in cancer gene therapy

Review Article

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Abbreviations: 5' untranslated region, (5'UTR); cationic amino acid transporter, (Cat-1); dihydrofolate reductase, (DHFR); hypoxia-inducible factor-1α, (HIF-1α); internal ribosome entry site, (IRES); methylguanine methyltransferase, (MGMT); multidrug-resistance 1 gene, (MDR1); open reading frames, (ORF); vascular endothelial growth factor, (VEGF)

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Summary

Cancer gene therapy is a promising treatment modality. Strategies in cancer gene therapy include tumor-directed therapy (e.g. the delivery of suicide, immunomodulatory, anti-angiogenic, apoptotic genes or oncolytic viruses or genes to reinstate tumor suppressor activity) and host-directed therapy (e.g. the delivery of genes encoding factors that enhance the antigen presenting function of dendritic cells or protect the patient against myelosuppression). As cancer, a complex disorder, often results from several defective genes, efficacy of cancer gene therapy can be improved by a combination approach whereby several different genes are targeted simultaneously. Of several methods to effect co-expression of multiple genes, the employment of internal ribosome entry sites (IRES) represents a promising approach. This review examines the various preclinical and clinical studies employing IRESs for cancer gene therapy, as well as properties of various IRESs that could be exploited for cancer gene therapy.

I. Introduction

Efforts to combat cancer with gene therapy have been underway for more than a decade (Gottesman, 2003), with several clinical trials having been conducted with varying success (Schuler et al, 2001; Buller et al, 2002; Kuball et al, 2002; Pagliaro et al, 2003). Because cancer pathogenesis stems in part from genetic mutations, gene therapy is, in concept, a viable approach to cancer treatment. Gene therapy is also of considerable utility on several fronts not directly pertaining to tumor-specific therapy, for example the delivery of drug resistance genes to mitigate myelotoxicity of chemotherapeutic agents.

II. Strategies in cancer gene therapy

A. Tumor-directed therapy

Fundamental tenets in cancer biology are that deregulated growth is due to a combination of the activation of oncogenes and inhibition of tumor suppressor genes, both of which present as obvious targets for cancer gene therapy. To date, most of the clinical trials have centered on reinstating tumor suppressor activity, in particular p53. However, the results concerning clinical efficacy have not been impressive (Zeimet and Marth, 2003; McNeish et al, 2004). One conceivable reason could be that modifying the expression of a single gene alone is insufficient to prohibit cancer growth because of numerous diverse pathways that still permit cancer progression. This, in theory, could be countered by the delivery of multiple genes that act on different pathways, such that a complementary or synergistic effect is obtained.

Other major themes in tumor-directed therapy include the delivery of suicide, immunomodulatory, anti-angiogenic, apoptotic genes and oncolytic viruses. Suicide genes encode enzymes that convert prodrugs to their cytotoxic form, and the herpes simplex virus thymidine kinase, which converts ganciclovir to ganciclovir phosphate, falls under this category. The immunomodulatory genes employed often code for cytokines, an example being interleukin 2, and these serve to mobilize the immune system to effect tumor cell killing. Strategies involving suicide and immunomodulatory genes are a popular combination in cancer gene therapy (Pizzato et al, 1998; Soler et al, 1999; Wen et al, 2001; Barzon et al, 2002).

Tumor cells actively induce the formation of new blood vessels, and a recent paradigm in oncology is the use of agents to impede this process, with a number of ongoing clinical trials evaluating the effectiveness of such agents. Gene therapy has been proposed to have several advantages over protein-based inhibitors, including the sustained expression of antiangiogenic molecules and the ability to deliver multiple transgenes (Kleinman and Liau, 2001).

The induction of apoptosis in cancer cells is another strategy, and studies involving the delivery of genes coding for pro-apoptotic factors, such as TRAIL, *Bax* and Smac/Diablo, have been conducted (Waxman and Schwartz, 2003). With an increasing recognition that most anticancer treatment modalities such as chemotherapy or radiotherapy trigger apoptosis of cancer cells, gene therapy may also prove useful in sensitizing the cells to the effects of conventional agents.

Oncolytic viruses selectively replicate in and kill tumor cells, and this specificity has contributed to their favorable safety profile. However, clinical trials have demonstrated an over-attenuation of these agents to the extent that efficacy has been compromised. Hence there has been a move to arm them with therapeutic genes to improve their tumor-killing capabilities (Hermiston and Kuhn, 2002).

B. Host-directed therapy

Myelosuppression is an extremely frequent complication of treatment utilizing conventional chemotherapeutic agents, and this at times may prove fatal. Hence a leading paradigm in cancer gene therapy is the delivery of genes to protect susceptible haemopoietic cells from the effects of these cytotoxic agents. Commonly employed drug-resistance genes include the multidrug-resistance 1 gene (*MDR*1), dihydrofolate reductase (*DHFR*) gene and methylguanine methyltransferase (*MGMT*) gene (Sorrentino, 2002).

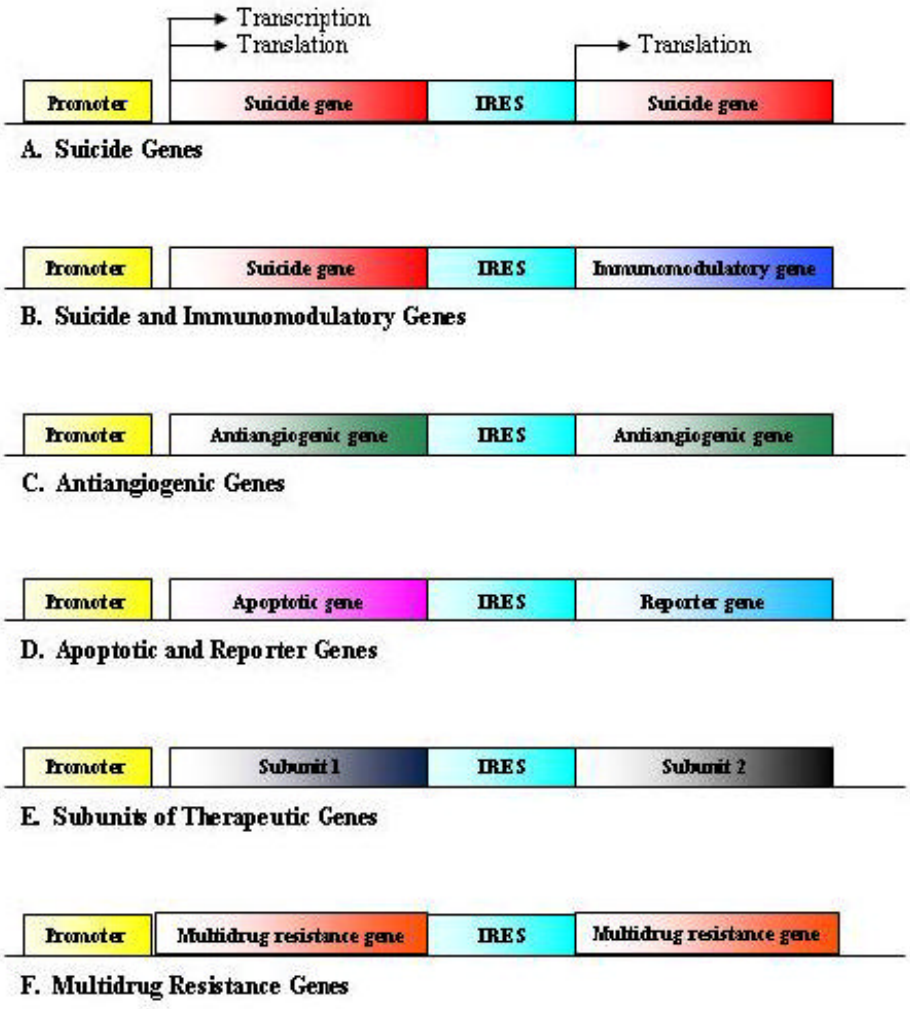

Figure 1. Strategies in Cancer Gene Therapy to date utilizing IRESs

Tumor vaccines are another promising modality (Berzofsky et al, 2004), and there are a variety of methods to induce tumor immunity. Naked DNA expression plasmids encoding tumor antigens have been shown to generate immune responses. Another approach is to deliver genes coding factors that enhance the antigen presenting function of dendritic cells.

III. Multiple gene delivery and attendant problems

As noted above, the ability to co-express multiple genes would be of immense value in cancer gene therapy because complementary or synergistic effects could lead to improved efficacy. Viruses are popular vectors for gene delivery because of their higher transduction efficiency, but this advantage is offset by the constraints placed on the vector size. Because most therapeutic genes are quite large, a polycistronic vector must be designed in such fashion that the system of effecting multigene delivery is modest in scale.

There are several methods available to effect multiple gene expression. One could be the incorporation of multiple promoters such that different proteins are produced from separate mRNAs. A major drawback of this approach is the possibility of promoter suppression (Emerman and Temin, 1984), a phenomenon whereby expression of any gene may be attenuated for ill-defined reasons.

Other methods including splicing, fusion proteins and proteolytic processing have been reviewed by de Felipe (2002).

IV. Internal ribosome entry sites

In eukaryotes, initiation of translation of most mRNAs begins by a cap-dependent mechanism whereby a 43S complex (comprising a 40S subunit, the initiator methionine-tRNA and other initiation factors) is recruited to the 5' methylguanosine cap. Recognition of the 5' end is mediated through the cap-binding protein complex eIF4F, which comprises three subunits eIF4E, eIF4A and eIF4G subunits. The 43S complex then scans in a 5' to 3' direction until an initiation codon is encountered, following which the initiation factors dissociate and a larger 60S ribosomal subunit binds to form the 80S ribosome. Protein synthesis then commences.

IRESs are RNA structures capable of initiating ribosome binding and translation in the absence of a 5' cap. Most commonly found in the 5' untranslated region (5'UTR) of mRNAs, they were first documented in poliovirus and other viral RNA sequences (Pelletier and Sonenberg, 1988), but were subsequently shown to exist in cellular mRNAs as well. To date there have been more than 50 reported viral and cellular IRESs in total, and the list is steadily expanding. The subject of IRESs has been extensively reviewed, both in the academic (Hellen and Sarnow, 2001; Stoneley and Willis, 2004) and applied (Ngoi et al, 2004) setting.

In utilizing this system for multiple gene co-expression, an internal ribosome entry site (IRES) is placed between two or more open reading frames (ORF), such that a corresponding number of proteins are generated from a single mRNA transcript.

V. Application of IRESs in cancer gene therapy

IRESs have been employed in a number of preclinical and clinical studies with some success, and selected ones, that span the gamut of cancer gene therapy, are displayed in **Table 1**.

VI. Choice of IRES?

Most of the studies detailed in **Table 1** employ the EMCV IRES, but a number of studies have reported that other IRESs may possess greater activity than the EMCV IRES, for example the eIF4G IRES (Wong et al, 2002). IRESs display a huge variation in their activity in various contexts, and given the burgeoning number of IRESs, it might be possible to tailor an IRES for a particular purpose, for example in the treatment of a certain type of cancer. However, current data is too sparse to allow a meaningful decision making process as to the best IRES for a given tumor type. Some factors governing the choice of IRES are discussed, and **Table 2** displays known properties of IRESs that might be useful in developing an effective polycistronic vector.

A. Tissue/Cell type specificity

IRESs have not been shown to display a narrow tissue/cell type specificity, and therefore cannot be employed in situations where this property is requisite for expression of the 3' cistron, in contrast to tumor-specific promoters.

B. Tissue/Cell type activity

Unfortunately not much is know about the tissue / cell type specificity of the different IRESs. Most IRES studies have investigated the activity of a particular IRES in different cell types, but the most valuable information pertaining to gene therapy application can only be gleaned from studies that have compared the activity of different IRESs in a particular tumor type. Nevertheless, known properties of some IRESs are detailed in **Table 2.**

C. Milieu-dependent activity

Certain stressful conditions are known to suppress cap-dependent translation, for example hypoxia, starvation or apoptosis, leading to a general decrease in protein synthesis. In this regard, IRESs possess a theoretical advantage over other modalities such as promoters, because some IRESs continue to operate under such conditions - conditions that are typically experienced by tumor cells. For example, the vascular endothelial growth factor (VEGF) IRES (Stein et al, 1998) and hypoxia-inducible factor-1α (HIF-1α) IRES (Lang et al, 2002) maintain activity during hypoxia; and the cationic amino acid transporter (Cat-1) IRES (Fernandez et al, 2001) exhibits increased activity during amino acid starvation. Where an IRES, such as the BCL-2 IRES (Sherrill et al, 2004), displays increased activity following cytotoxic drug

Table 1. Preclinical and Clinical Studies to date utilizing IRESs

Preclinical Studies (Tumor-directed therapy)								
Year published	**Strategy/Aim of Study**	**IRES employed**	**Therapeutic/market/reporter genes encoded**	**Vector**	**Cell Lines**			**References**
2004	Arming an oncolytic virus with a suicide gene	EMCV	yCD	Human adenovirus 5	1. SW480	Colon cancer	Human	(Fuerer and Iggo, 2004)
					2. HCT116	Colon cancer		
					3.HT29	Colon cancer		
	Suicide gene delivery	EMCV	1. P450 2. NADPH-cytochrome P450 reductase	Replication-defective adenovirus	1. A549	Lung cancer	Human	(Jounaidi and Waxman, 2004)
					2. EKVX	Lung cancer		
					3. HT29	Colon cancer		
					4. IGROV1	Ovarian cancer		
					5. MDA-MB-231	Breast cancer		
					6. MDA-MB-435	Breast cancer		
					7. NCI-H226	Lung cancer		
					8. NCI-H522	Lung cancer		
					9. PC-3	Prostate cancer		
					10. RXF-393	Renal cancer		
					11. T47-D	Breast cancer		
					12. U251	Glioblastoma multiforme		
					13. 786-0	Renal cancer		
	Fusion of reporter gene to various oncolytic viral genes	EMCV	Luciferase reporter gene	Conditionally replicative adenovirus	1. A549	Lung cancer	Human	(Rivera et al, 2004)
	Antiangiogenesis	EMCV	1. Angiostatin 2. Endostatin 3. GFP	Recombinant adenovirus-associated virus	1. 293	Embryonic kidney	Human	(Ponnazhagan et al, 2004)
					2. SKOV3.ipl	Ovarian cancer		
2002	Charecterization of activity of different IRESs in varying contexts using reporter assays	1. EMCV 2. BIP 3. eIF4G 4. MYC 5. VEGF	1. CAT 2. GAL	Plasmid	1. KB-3-1	Cervical cancer	Human	(Wong et al, 2002)
					2. 293	Embryonic kidney		
					3. HepG2	Liver cancer		
					4. N2a	Neuroblastoma	Mouse	
	Suicide and immunomodulating gene delivery	EMCV	1. HSV-tk 2. IL-2	Retrovirus	1. WRO	Thyroid cancer	Human	(Barzon et al, 2002)
					2. FTC-133	Thyroid cancer		
					3. C8305	Thyroid cancer		
					4. ARO	Thyroid cancer		
					5. HeLa	Cervical cancer		
					6. AoU373	Astrocytoma		
					7. HepG2	Liver cancer		
	Induction of apoptosis	EMCV	1. TRAIL 2. GFP	Adenovirus	1. Cwr22Rv1	Prostate cancer	Human	(Voelkel-Johnson et al, 2002)
					2. Dul45	Prostate cancer		
					3. DuPro	Prostate cancer		
					4. JCA-1	Prostate cancer		
					5. LNCaP	Prostate cancer		
					6. PC-3	Prostate cancer		

					7. PPC-1	Prostate cancer		
					8. TsuPr1	Prostate cancer		
					9. PrEC	Primary prostate epithelial cells		
2001	Immunotherapy	1. EMCV 2. FMDV	1. IL-12p40 2.IL-12p35 3. CD80	1. Retrovirus 2. Adenovirus	1. U266	Myeloma	Human	(Wen et al, 2001)
					2. OCI-My5	Myeloma		
					3. ANBL-6	Myeloma		
					4. K562	Leukemia		
					5. Namalwa	Myeloma		
1999	Tumor cell vaccine	EMCV	1. HSV-tk	Retrovirus	1. 9L	Gliosarcoma	Rat	(Okada et al, 1999)
			2. IL-4					
			3. Neomycin					
			4. phosphotransferase					
1998	Suicide and immunomodulating gene delivery	EMCV	1. IL-2	Retrovirus	1. Al72	Glioblastoma	Human	(Pizzato et al, 1998)
			2. HSV-tk		2. AoU373	Astrocytoma	Human	
Preclinical Studies (Host-directed therapy)								
2001	Myeloprotection	EMCV	1. ALDH-1	Retrovirus	1. NIH3T3	Fibroblast	Mouse	(Takebe et al, 2001)
					2. Primary $CD34^+$ cells		Human	
1999	Myeloprotection and cell-surface marking	EMCV	1. MDR1 2. ΔLNGFR	Retrovirus	1. K562	Leukemia	Human	(Hildinger et al, 1999)
					2. Primary $CD34^+$ cells			
Year published	**Strategy/Aim of Study**	**IRES employed**	**Therapeutic/marker/reporter genes encoded**	**Vector**	**Tumor type**			**References**
1999	Suicide and immunomodulating gene delivery	EMCV	1. IL-2	Retrovirus	Glioblastoma mulriforme			(Palu et al, 1999)
			2. HSV-tk					

ALDH-1 (aldehyde dehydrogenase), CAT (chioramphenicol acetyltransferase), F/S DHFR (doubly mutated dihydrofolate reductase), GAL (beta-galactosidase), GFP (green fluorescent protein), HSV-TK (herpes simplex virus thymidine kinase), IL2 (interleukin 2), IL 12 (interleukin 12), ΔLNGFR (truncated human low-affinity nerve growth factor receptor), yCD (yeast cytosine deaminase)

Table 2. Known properties of some IRESs

IRES	**Properties**	**Cell lines**			**References**
BCL-2	Reported to exhibit 3.4-fold greater activity following 8h treatment with 80μM etoposide compared to untreated cells.	**1. 293T**	Embryonic kidney	Human	(Sherrill et al, 2004)
Cat-1	Reported to exhibit 7-fold greater activity following 12h amino acid starvation compared to fed cells. Activity compared to the EMCV TRES unknown	1. C6	Glioma	Rat	(Fernandez et al, 2001)
Connexin43	Reported to exhibit 18-fold greater activity than the EMCV IRES.	1. HeLa	Cervical cancer	Human	(Schiavi et al, 1999)
DAP5	Reported to exhibit at least 2-fold greater activity than the EMCV IRES following 48h etoposide treatment.	1. 293T	Embryonic kidney	Human	(Nevins et al, 2003)
eIF4G	Reported to exhibit at least 200-fold greater activity than the EMCV IRES	1. KB-3- 1	Cervical cancer	Human	(Wong et al, 2002)
		2. HepG2	Liver cancer	Human	
Gtx	9-nucleotides in length. 10 linked copies reported to exhibit 63-fold greater activity than the EMCV IRES.	1. N2a	Neuroblastoma	Mouse	(Chappell et al, 2000)
HIF- 1α	Activity maintained during hypoxia. Activity compared to the EMCV IRES unknown.	1. NIH3T3	Fibroblast	Mouse	(Lang et al, 2002)
N-myc	Reported to exhibit 5-7 fold greater activity than the c-myc IRES.	1. NB2a	Neuroblastoma	Mouse	(Jopling and Willis, 2001)
		2. SH-SY5Y	Neuroblastoma	Human	
	3-fold greater activity compared to the EMCV IRES.	3. HeLa	Cervical cancer	Human	
VEGF	Activity maintained during hypoxia. Activity compared to the EMCV IRES during hypoxia unknown.	1. C6	Glioma	Rat	(Stein et al, 1998)

administration, the design of therapeutic regimes to exploit this property, for example to augment cytotoxicity, is conceivable.

D. Size

Most IRESs tend to be relatively large, and this may limit the number of transgenes that can be incorporated into a polycistronic vector. A 9-nucleotide long IRES residing in the 5'UTR of the Gtx homeodomain RNA has been reported (Chappell et al, 2000), and appears to function in a modular fashion, such that multiple linked copies increase the expression of the downstream cistron. Besides the advantages of its small size, it also allows for regulated expression of the downstream cistron by varying the number of intercistronic modules.

VII. Current problems with IRESs in gene therapy

A traditional problem concerning the use of IRESs is that expression levels of the gene downstream of the IRES is often significantly lower than that of the upstream gene, typically around 20-50% (Mizuguchi et al, 2000) in bicistronic plasmid vectors in relation to the upstream gene, and even lower in retroviral vectors (de Felipe, 2002). Another major stumbling block is the inconsistency of gene expression depending on the composition and arrangement of genes in the vector (Hennecke et al, 2001).

VIII. Future directions

The vast majority of cancers result from defects in multiple pathways, and hence an effective gene therapeutic approach will probably have to be multi-pronged, requiring delivery of different transgenes that target the different pathways. The studies detailed in **Table 1** have demonstrated proof of concept for employing IRESs to effect the co-expression of multiple genes in diverse fields of cancer gene therapy. As noted above more information concerning the activity of various IRESs in a tissue/cell-type, both *in vivo* and *in vitro*, is required to facilitate decision-making in the choice of IRES. It is envisaged that the incorporation of IRESs with desirable properties will result in polycistronic vectors with improved downstream gene expression, and consequently result in enhanced clinical efficacy.

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