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Increasing Tumour Immunogenicity by Genetic Modification

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INTRODUCTION

IT IS GENERALLY believed that tumour growth in most cases results from: (1) the accumulation of mutations in genes which alter cell differentiation and proliferation (e.g. oncogenes, tumour suppressor genes); and (2) the failure of the immune system to recognise and/or destroy those cells which are behaving abnormally. Although a large number of mutant cellular genes have been identified in tumour cells, it is apparently unpredictable when they occur and to detect them at early stages of tumour growth. Therefore, it is understandable that much interest has focused on developing new strategies in order to activate the host's immune system against tumours. This concept has a long history and has been characterised by Oettgen and Old [1] as 'a field that has gone through recurring cycles of enthusiasm and disillusionment'. In other words, none of the many approaches of cancer immunotherapy has fulfilled the hopes raised by well-defined experimental animal systems once they were employed in patients.

The subject is still highly relevant, however. Firstly, traditional forms of cancer therapy, such as surgery, radiation therapy and chemotherapy, although effective in many instances, often fail to provide long-term cure for cancer. Secondly, significant knowledge of regulation of immune responses and the mode of antigen presentation to T-cells allows the development of new strategies. Furthermore, many investigators involved in clinical trials report on individual but isolated cases of complete tumour regression, which appear to correlate with some form of immune intervention. However, the reasons for occasional tumour regression remain obscure.

Broadly speaking, specific and non-specific strategies of cancer immunotherapy can be distinguished. The former mainly involved agents such as 'Coleys toxin' or adjuvants such as Bacillus Camette-Guerin (BCG) and *Corynebacterium parvum*. A large number of clinical trials have been carried out (reviewed in [1]), and they are mentioned here only because, at least in one clinical situation, significant therapeutic effects have been obtained in randomised trials. This was achieved by intravesical BCG instillation in superficial bladder cancer patients [2], although the underlying mechanism is not known. Specific cancer immunotherapy strategies can rely on tumour-reactive antibodies, adoptive transfer of immune cells (mainly LAK cells or T lymphocytes), tumour cells (modified or non-modified; autologous or allogeneic) as vaccines or tumour-specific antigens (peptides) as vaccines. The term *vaccine* usually refers to a therapeutic rather than a prophylactic modality. All approaches are based on the assumption that tumour cells can be recognised specifically by immune cells or their products. The possibility of raising tumour-specific cytotoxic T lymphocytes (CTL) against mouse and human tumours [3–5], the recent cloning of genes encoding antigens recognised by CTL [6–8] and the fact that tumours engineered to secrete a single additional soluble protein can be rejected [9–11], strongly support the assumption that most, if not all, tumours possess antigens against which an immune response can be triggered. Although evidence demonstrating that *in vitro* defined tumour antigens may serve as rejection antigens *in vivo* is still lacking [12], and tumour antigens must not necessarily be tumour specific [7] which, in the case of successful immunotherapy, could result in autoimmunity. These results have once again revived optimism that any of the above-mentioned strategies for cancer immunotherapy will find its successful clinical application.

Pertinent to this review are tumour cell vaccine strategies only. Antibody [13], immune cell [4, 14, 15] or peptide vaccine strategies [16] have been reviewed recently. Extensive clinical experience exists with tumour cell vaccines, including irradiated autologous or allogeneic tumour cells injected as vaccine, tumour cell lysates, fractions of tumour cells, virus or chemically-modified tumour cells and tumour cells plus adjuvant (mostly BCG or *C. parvum*). Many different tumours have been tested. Considering this clinical experience, Oettgen and Old [1] have drawn the conclusion that 'these trials did not produce convincing evidence of therapeutic efficacy when they were appropriately controlled' (with the possible exception of one colon cancer study using autologous tumour cell/BCG as vaccine) [17]. Therefore, it is not surprising that there is an enormous demand for more effective and more precise vaccines. One option, currently being investigated in a number of laboratories, is based on using cytokines as highly immunologically active agents and modern techniques of molecular genetics in order to genetically engineer tumour cells. A variety of different cytokines have been expressed in tumour cells in order to study whether, upon injection into mice, an increased local cytokine concentration results in a change of tumorigenicity. As will be shown in this review, a surprisingly large number of cytokines are able to induce a local immune response, leading to tumour rejection. The immunogenicity rather than the antigenicity of the tumour should have been changed by the secreted cytokine, which raises the question as to whether such genetically modified cells also induce systemic (T-cell-mediated) immunity, of sufficient potency to recognise and destroy distantly-growing non-modified tumour cells, and whether potency is improved over traditional vaccine regimens. The tumorigenicity and immunological effects of cytokine gene transfected tumours will be reviewed, followed by a discussion of the key findings concerning the vaccination potential of such cells.

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LOCAL EFFECTS WITH CYTOKINE GENE TRANSFECTED RODENT TUMOUR CELLS

Interleukin-1 (*IL*-1)

Douvdevani and colleagues have transfected the IL-1α gene into oncogene -transformed fibroblasts and also selected spontaneously IL-1α producing cells [18]. Unlike the parental cells, IL-1 secreting cells were rejected in immunocompetent mice. IL-1 was not active in T-cell deficient (nude) mice and cytotoxic T-cells have been implicated in the rejection process.

IL-2

Based on the many clinical trials of systemically administered IL-2, that showed some objective responses which were usually accompanied by toxic side-effects, a logical step was to analyse the consequences of increased local IL-2 concentration close to the tumour site.The IL-2 gene has been expressed in mouse tumour cell lines X63-Ag8 [19], CT26 [20], B16-F10 [20, 21], CMS-5 [22], P815 [23], TS/A [24], J558L [25], MCA-102 [26], 3LL-D122 [27], MBT-2 [28], 4T07 [29], LLC [30] and in rat tumour cell lines HSLNV [31] and RAT-1 [32]. A consistent observation was that IL-2 secretion resulted in decreased or, dependent on the level of IL-2 production, loss of tumorigenicity. IL-2-secreting cells suppressed the growth of co-injected parental tumour cells [22, 32]. T-cell-dependent and -independent effector mechanisms were responsible for tumour rejection. Cytotoxic CD8+ T cells, but not CD4+ T cells, were needed for complete tumour eradication [20, 25], and for the prevention of outgrowth of IL-2 loss variants [25]. As shown below, IL-2 shares these features with several other cytokines. The non-T-cell effector arm seems to be mediated largely by natural killer (NK, asialo GM1+) cells [25, 26], but immunohistology has detected macrophages [25] and, in another system, neutrophils [24] in IL-2-producing tumours. It is a frequent observation that the same cytokine transfected into different tumour cells yields varying or even opposite effects. It is important to note that, in several studies, IL-2 expression vectors that encoded additional viral proteins have been used [19, 20, 23, 24]. Firstly, contaminant helper virus can induce tumour rejection independent of IL-2 expression [31], and secondly, only co-expression of both a neo-antigen and an immunostimulatory molecule (the T cell co-stimulatory molecule B7), but not either gene alone, led to tumour rejection [33]. Thus, introduction of viral sequences into tumour cells may contribute to the effects attributed to the cytokine.

IL-4

Tepper and colleagues showed that IL-4 gene transfected tumour cells were rejected effectively [34]. This effect was immunologically mediated and correlated with a macrophage and eosinophil infiltrate in IL-4-producing tumours. Consistently, cells grown in an IL-4 autocrine fashion, following retroviral IL-4 gene transfer, did not become tumorigenic in contrast to observations with several other cytokines in analogous experiments [35]. The anti-tumour efffect of IL-4 operates in T-cell-deficient mice but, at least for cells producing less IL-4, CD8+ T-cells were required for complete tumour eradication [25, 36]. Meanwhile, with IL-4 gene transfer, it has been shown that tumour growth of J558L [25, 34, 36], K485 [34], RENCA [37], B16-F10 [21], CHO [38] and LLC cells [30] was suppressed. In order to analyse the cascade of events induced by tumour cell-derived IL-4, host cytokines have been identified by means of a quantitative PCR assay during the early phase of the anti-tumour response against IL-4-secreting CHO cells in nude mice [38]. Correlating with tumour IL-4 and host IL-4 receptor mRNAs, IL-5 and IFN-γ mRNAs were detected and an anti-IFN-γ antibody was shown to partially restore tumour growth of CHO-IL-4 cells. Similar PCR results were obtained in IL-4 transgenic mice [39]. The anti-tumour effect of IL-4 could be reversed partially by an antibody directed against eosinophils [40]. It is possible that together, eosinophils and IFN-γ-induced macrophages mediate the T-cell-independent tumour suppression induced by IL-4.

IL-5

Using retroviruses, the IL-5 gene was introduced into two IL-5-dependently growing cell lines [41]. Correlating with growth autonomy caused by the endogenously produced IL-5, the 'autocrine' growing cells, but not the mock-transfected cells, gave rise to tumours in nude mice. Expression of the IL-5 gene in different amounts in tumour cells J558L, TS/A [42] and B16-F10 [21] did not result in a change of tumorigenicity in syngeneic mice, although in the first two cases, tumours were heavily infiltrated by eosinophils. Because one of the cell lines (J558L) was the same by which an eosinophil-dependent mechanism of the anti-tumour effect of IL-4 has been shown [40], these results demonstrate that the presence of immune cells *per se* does not allow conclusions to be drawn about the contribution of the cells to tumour suppression, and that appropriate activation of tumour infiltrating cells is essential.

IL-6

IL-6 has been implicated in the support of tumour growth. For example, IL-6 was shown to be constitutively expressed in a plasmacytoma cell line as a result of an IL-6 gene rearrangement [43], and conferred increased tumorigenicity to cells grown in an autocrine fashion following IL-6 gene transfer [44]. IL-6-expressing J558L cells were not retarded in tumour growth [45], and no infiltrating cells were detected in the tumours [25]. B16-F10 cells transfected to produce IL-6 showed only a moderate delay in tumour formation [21], and reduced experimental metastases attributed to non-specific inflammatory mechanisms [46]. In one tumour cell line (3LL-D122), IL-6 expression caused growth inhibition *in vivo* that correlated with growth arrest *in vitro* [47]. Thus, IL-6 has very different or even opposite effects on tumour growth compared with other cytokines.

IL-7

IL-7 very effectively suppresses tumour growth. This has been demonstrated by gene transfer and expression of IL-7 in tumour cell lines J558L, TS/A [25, 48], FSA [49] and 203-glioma [50], which consistently resulted in loss of or, depending on the amount of secreted IL-7, reduced tumorigenicity. This effect was the result of a complex immunological reaction. CD4+ and CD8+ T-cells accumulated independently in IL-7-producing tumours. Additionally, CD4+ T-cells caused infiltration by macrophages and eosinophils, possibly by secondary cytokines, which still have to be identified. Tumour suppression by IL-7 was entirely dependent on T-cells [48]. This was not the case with other cytokines, such as IL-2, IL-4, TNF or IFN-γ, analysed in the same tumour model [25]. CD4+ T-cells and recruited macrophages (and possibly eosinophils whose involvement in tumour suppression still has to be proven) were required to prevent rapid tumour burden, but in most cases were unable to completely eradicate the tumour. In contrast, CD8+ T-cells were not involved in the early phase of tumour suppression but were imperative for long-term tumour eradication. In this

regard, IL-7 did not differ from IL-2, IL-4, TNF and IFN-γ[25]. Although IL-7 reliably suppressed several transfected tumours, the precise mechanism of rejection induced by IL-7 can be influenced by the particular tumour [50]. A more detailed review of effects with IL-7 gene transfected tumours has recently been published [51].

IL-10

IL-10 has immune suppressive and anti-inflammatory activities in several experimental systems. Therefore, it was surprising to learn that CHO cells engineered to secrete IL-10 had completely lost the ability to grow as tumour in nude or SCID mice [52]. Since CHO, but not CHO-IL-10 tumours, were heavily infiltrated by macrophages, it was suggested that the macrophages in CHO tumours provide a paracrine tumour growth promoting activity which was downregulated by IL-10. It is not yet known which factor(s) CHO cells require from macrophages, however, the fact that CHO-IL-10 cells were impaired to establish early tumour growth, raised the possibility that angiogenetic factors were required from the macrophages, a hypothesis which has yet to be proven. Another tumour cell line (TS/A), transfected to express similar levels of IL-10, was not growth inhibited *in vivo* (unpublished). In contrast to other cytokines which induce tumour suppression by immunostimulation, IL-10 seems to mediate its anti-tumour activity on some tumours by local suppression of immune function.

Tumour necrosis factor (TNF)

A variety of different tumour cell lines have been transfected or retrovirally infected with TNF gene-containing vectors: ovarian carcinoma CHO [53], plasmacytoma J558L [45], UV-induced skin tumour 1591-RE [54], sarcomas MCA-205 [55] and MCA-102 [26], T-lymphomas EB and ESB [56] and melanoma B16-F10 [21]. With the exception of one tumour cell line which grew more slowly *in vitro* after TNF gene transfer [26], TNF expression did not change the growth characteristics of the cells *in vitro*. Injection of the TNF-producing cells into mice revealed that most of the tumour lines were growth inhibited in a TNF dose-dependent fashion, without systemic toxicity. A complex pattern of tumour-infiltrating cells has been observed in TNF-producing tumours consisting of macrophages, CD4+ and CD8+ T-cells [25, 45]. For tumour suppression, macrophages [45] and CD8+ T-cells [25] were needed. A contribution of CD4+ T-cells has been shown in some [55] but not in other models [25]. TNF was active in T-cell-deficient mice, but in most cases, T-cells were needed for complete tumour elimination. Recent experiments also have shown that local TNF failed to induce an anti-tumour response against certain tumours [26, 56]. Moreover, depending on the tumour cell line which was used or on the level of TNF secreted by the tumour, systemic toxicity has been observed leading to cachexia or wasting of the mice [21, 53, 54]. In one case, it has been shown that a TNF gene-transfected tumour showed no tumour growth inhibition *in vivo*, but TNF augmented metastases of these cells [56]. These observations with TNF gene transfected tumours have been reviewed recently [57].

Interferon (IFN)-α

Friend leukaemia cells (FLC), transfected to produce IFN-α, have been shown to be rejected in immunocompetent but not in nude mice, suggesting immune-mediated rejection involving T-cells [58].

IFN-γ

Tumour cells C1300 [59], CMS-5 [60], SP1, CT26 [61], MCA101 [62], J558L [25], B16-F10 [21], MBT-2 [28] and 3LL-D122 [63] have been transfected with the IFN-γ gene. Strong tumour suppressive effects have been observed in most cases of relatively high IFN-γ expression [25, 59, 60, 61, 63], while low levels of IFN-γ secretion have resulted in moderate [21] or no delay of tumour formation [62]. The mechanism of rejection is not entirely consistent in the different models. In several cases, increased expression of MHC class I molecules on tumour cells has been observed, for which low (non-tumour-suppressive) amounts of IFN-γ production were sufficient [59–63]. Cytotoxic CD8+ T-lymphocytes have been implicated in tumour rejection in some [25, 59, 60, 63] but not other models [61]. In addition, IFN-γ was operative in nude mice in some [25, 63] but not other tumour systems [61]. NK (asialo GM1+) cells can contribute to tumour rejection, and macrophages, whose function is not known, have been detected in IFN-γ-producing tumours [25]. An interesting observation was that IFN-γ producers, which developed after a long latency period in nude mice, still produced undiminished amounts of IFN-γ, in contrast to analogous experiments with IL-2, IL-4 and TNF expressed in the same tumour and grown with similar kinetics in nude mice [25].

Granulocyte colony-stimulating factor (G-CSF)

G-CSF has been expressed in C26 tumour cells, resulting in loss of tumorigenicity [64]. As expected from the restricted activity of G-CSF, neutrophils appear to be the only effector cells. Subsequent studies showed that large tumours, raised in syngeneic mice that had been transiently immunesuppressed, were suppressed by irradiation, regressed due to secondary, granulocyte-dependent mechanisms, which involved CD8+ T-cells and IFN-γ [65].

Macrophage (M)-CSF

J558L tumours transfected to produce M-CSF were heavily infiltrated by macrophages, whereas the control tumours were not [66]. However, tumour formation was only slightly retarded. Thus, despite its chemotactic activity on macrophages, M-CSF alone was not sufficient to activate macrophages for tumoricidal activity. It should be noted that the same tumour, producing either TNF or IL-7, was similarly infiltrated by macrophages which actually contributed to tumour rejection [25, 45, 48].

GM-CSF

GM-CSF conferred tumorigenicity to factor-dependent cells following transfer of the gene responsible [67]. Likewise, GM-CSF gene transfected B16 F10cells grew progressively without delay compared with parental cells and induced severe toxicity [21].

Macrophage chemotactic and activating factor (MCAF)

The cytokine MCAF (MCP1, JE) selectively acts on macrophages. Expression of MCAF in CHO cells abrogated tumorigenicity in nude mice which correlated with a monocytic tumour infiltrate [68]. An increase in tumour infiltrating macrophages was also observed in MCAF-producing B16 cells. However, these cells grew as tumour, albeit with some delay [69].

IP-10

IP-10 belongs to the structurally-related cytokine gene family also known as chemokines. Other members include IL-8, GRO, PF-4 and β-thromboglobulin. Expression of IP-10 in cell lines

J558L and K485 abrogated their tumorigenicity [70]. Interestingly, the function of IP-10 was unknown prior to this assay, but the experiment showed IP-10 to induce a strong inflammatory response by lymphocytes, neutrophils and monocytes. Tumour rejection by IP-10 was shown to involve T cells.

SYSTEMIC EFFECTS WITH CYTOKINE GENE TRANSFECTED TUMOUR CELLS

The high efficacy of transfected cytokines in inducing rejection of gene modified tumours and the frequently-observed contribution of T-cells to tumour rejection, raises questions such as: are cytokine gene transfected tumour cells useful as vaccines and which genetic manipulations are most suitable for inducing systemic tumour immunity? In several tumour models, it was shown that mice which had rejected a cytokine-producing tumour, resisted a subsequent challenge with parental tumour cells. However, this effect could not be attributed conclusively to the transfected cytokine rather than to the inherent immunogenicity of the tumour cells [21]. More recent results have defined the contribution of transfected cytokines to the generation of protective immunity.

(1) In several tumour models (presumably minimally- or non-immunogenic), it has been shown that immunisation with cytokine (IL-2, IL-4, IL-6, IL-7, TNF, IFN-γ, GM-CSF)-producing tumour cells (live or irradiated) protected more mice from a subsequent tumour challenge than irradiated parental tumour cells [21, 24, 27, 28, 47, 63, 71]. However, as demonstrated in one tumour model, this effect was less obvious with higher challenge doses [71].

(2) No combination of IL-2, IL-4, IL-7, TNF or IFN-γ-producing cells revealed a synergistic effect [71].

(3) The observation that IL-2, IFN-γ or GM-CSF-producing tumour cells effectively vaccinated mice against either a subsequent tumour challenge, an established small tumour (1–2 weeks old) or metastasis [21, 24, 27, 28, 63], but that at least IL-2 and IFN-γ failed in other tumour models [26, 28], suggests that they are unlikely to find a place as a universal vaccination strategy. The high vaccination efficacy of GM-CSF producing tumours [21] needs to be investigated in more detail.

(4) A comparison of different cytokines is hampered by the problem that the vaccination effect may follow a dose–response relationship. Thus, tumour cells, producing varying amounts of IL-2, induced tumour immunity in a bell-shaped fashion with intermediate levels of IL-2 secretion being most effective [24].

(5) For evaluation of the clinical value of cytokine-producing tumour cells as vaccine, it seems more relevant to compare them with well-established vaccine strategies, such as mixtures of tumour cells plus adjuvant. In one comparative study, we showed that a tumour cell/*C. parvum* mixture was as effective in raising a protective response as IL-2, IL-4, IL-7, TNF or IFN-γ-producing cells, which suggests that genetically engineered tumour vaccines can still be improved [71]. Notably, live tumour cells were more effective than irradiated ones.

Recently, it was shown that transfection of B7 in tumour cells stimulated a CD8+ T-cell-mediated immune response, leading to tumour rejection and protective immunity [33, 72]. B7 is expressed mainly on antigen-presenting cells and serves as a co-stimulatory signal for T-cells, by interacting with its ligands CD28 or CTLA-4. It will be interesting to compare the two strategies and to analyse whether co-expression of cytokines and molecules, such as B7, in tumour cells can act synergistically to enhance tumour immunogenicity.

CONCLUSION

Localised cytokines, close to the tumour site, can induce a very effective immune response leading to tumour rejection without toxicity. By cytokine gene transfer into tumour cells, it has been shown that a considerable number but not all cytokines have anti-tumour activity. The immunological pathways induced by various cytokines which mediate tumour rejection have partially been resolved. A frequent observation is that T-cell-dependent and -independent mechanisms are activated and partially overlap for different cytokines. The anti-tumour effect of cytokines and the cellular mechanism of rejection depends on several variables, such as the tumour cell line or level of cytokine secretion. Experiments designed to demonstrate the vaccination potential of cytokine gene modified tumour cells are encouraging, but not yet ideal, since the tumour antigens against which the immune response is triggered have still to be identified.

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Clinical Application of Immunostimulatory Gene Transfer

B. Gansbacher

INTRODUCTION

Despite significant progress in the treatment of leukaemias, non-Hodgkin's lymphomas and Hodgkin's lymphoma, there is still much improvement to be made with chemotherapy for solid tumours. This can be illustrated by the fact that, in 1991, approximately 165 000 Americans developed lung cancer and, despite receiving optimal therapy with chemotherapy or radiation therapy, 143 000 died the same year. Similar observations have been made for many other solid tumours. Chemotherapy and radiation therapy have improved survival rates significantly, but new therapeutic strategies are required.

In 1980, several groups began developing new concepts to treat cancer which were based on activation of the immune system. It was known that effector cell populations of the immune system can kill tumour cells *in vitro*. Lymphocytes, natural killer cells, macrophages or lymphokine activated killer (LAK) cells, when co-cultured in a test tube with tumour cells, will kill the cancer cells under certain conditions. Discussion rapidly took place regarding manipulation of the effector cells *in vivo*. The fact that some of the effector cells expressed interleukin-2 (IL-2) receptors led to several studies in which systemic IL-2 was used in an attempt to mobilise and activate them.

Over the last 10 years, IL-2 has been used extensively in both animal models and cancer patients; it has been injected intravenously into lymph nodes or directly into tumours. However, side-effects have been common, and the results unimpressive. Partly to compensate for the short-half life of IL-2, megadoses have been given. Several patients treated in this manner have required intensive care and some have died. By 1989, it was clear this was not the optimal method of stimulating effector cell populations *in vivo* and new modalities were needed. It was believed that cytokines were necessary at the site, where effector cells encounter the tumour, because they were known to drive the differentiation and development of functional abilities of relevant effector cells. By using retroviral vectors, DNA transfection, adenoviruses and lipofection, several groups introduced cytokines directly into tumour cells and in this way forced them to secrete the cytokine directly at the tumour site. The cytokines included IL-2, IL-3, IL-4, IL-6, IL-7, granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), tumour necrosis factor (TNF), and many others [1].

Several cytokines have induced rejection of primary tumours in murine models. The effects on the immune system, induced by various cytokines, were compared with each other. Despite the attempt by some investigators to claim that they had found

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