Le morte du tumour: Histological features of tumor destruction in chemo-resistant cancers following intravenous infusions of pathotropic nanoparticles bearing therapeutic genes

ERLINDA M. GORDON[3,6], MARIA TERESA CHAN[1,3], NELSON GERALDINO[1,3,5], FRANCISCO F. LOPEZ[2-4], GERARDO H. CORNELIO[2-5], CONRADO C. LORENZO III[2-4], JOHN P. LEVY[6], REBECCA A. REED[6], LIQIONG LIU[6] and FREDERICK L. HALL[6]

Departments of [1]Pathology and [2]Internal Medicine/Medical Oncology; [3]Asian Hospital and Medical Center; [4]Makati Medical Center; [5]University of Philippines, Manila, Philippines; [6]Epeius Biotechnologies Corp., San Marino, CA 91108, USA

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Abstract. The pathotropic targeting of therapeutic nanoparticles to cancerous lesions is an innovative concept that has recently been reduced to practice in clinical trials for the treatment of metastatic cancer. Previously, we reported that intravenous infusions of Rexin-G, a pathotropic nanoparticle (or vector) bearing a cyto-ablative construct, induced tumor regression, reduced tumor burden, and improved survival, while enhancing the overall quality-of-life of patients with otherwise intractable chemotherapy-resistant cancers. In this report, we describe the major histopathological and radiologic features that are characteristic of solid tumors under the destructive influences of Rexin-G administered as a single therapeutic agent. To further promote tumor eradication and enhance cancer survival, we explored the potential of an auxiliary gene transfer strategy, specifically intended to induce a localized cancer auto-immunization in addition to assisting in acute tumor destruction. This immunization strategy uses Rexin-G in combination with Reximmune-C, a tumor targeted expression vector bearing a granulocyte macrophage-colony stimulating factor (GM-CSF) gene. Intravenous infusions of Rexin-G were given first to induce apoptosis and necrosis in the metastatic tumor nodules, thus exposing tumor neo-antigens, followed by Reximmune-C infusions, intended to recruit immune cells discretely into the same compartments (or lesions). The intent of this two-step approach is to bring a complement of cells involved in humoral and cell-mediated immunity in close proximity to the immunizing tumor antigens in a concerted effort to assist in tumor eradication and to promote a cancer vaccination *in situ*. Herein, we also describe the distinctive histopathologic and immunocytochemical features of tumors in terminal cancer patients who received Rexin-G infusions in combination with Reximmune-C. In addition to documenting the first histological indications of clinical efficacy achieved by this novel personalized approach to cancer vaccination, we discuss new methods and strategies for advancing its therapeutic utility. Taken together with the clinical data, these histological studies serve as valuable landmarks for medical oncology, and as definitive benchmarks for the emerging field of cancer gene therapy.

Correspondence to: Dr Erlinda M. Gordon, Epeius Biotechnologies Corp., 475 Huntington Drive, San Marino, CA 91108, USA
E-mail: emgordon@epeiusbiotech.com

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Introduction

The proverbial 'War on Cancer' is often declared and supported and discussed in vague socio-economic terms - Nixon's war, Run-for-the-Cure, Blockbuster Drugs - yet the battle is invariably waged on a much more personal level: the level of flesh, and blood, and sinews. This paper is a study of these tissues *in extremis*. A medical 'cure' for cancer (defined by long-term survival) is generally considered possible only in patients whose tumors are histologically localized to the extent that they can be completely resected by surgical methods. However, in patients with metastatic cancer, there is currently no effective therapy, and successful treatment is frequently directed at prolonging life or temporarily improving the general quality-of-life. Therefore, novel therapeutic modalities are urgently needed to create the histological and/or physiological conditions that can address the problem of metastatic spread and increase the feasibility of achieving an eventual cure. In this regard, the advent of pathotropic (or disease-seeking) targeting represents a crucial enabling technology (1,2). Indeed, the development of pathotropic nanoparticles that could serve as efficient gene delivery vehicles (or vectors) - vectors that are capable, by design, of seeking out, accumulating in, and destroying metastatic tumor nodules, no matter how remote and occult the location, while sparing normal cells and tissues, remains an all too elusive *beau ideal* of modern-day bioengineering (3-6).

Once regarded as a merely theoretical construct - an academic exercise in abject optimism, at best - the precision targeting of just such therapeutic nanoparticles directly to

cancerous lesions that have spread beyond the field of the most gifted surgeon, beyond the reach of the finest catheter, has finally been accomplished (1,2,7,8), and the safety and efficacy of these tumor-targeted nanoparticles in treating metastatic disease has been proven in the crucible of clinical medicine (9,10). The world's first such tumor-targeted nanoparticle, bearing a lethal dominant-negative form of the cyclin G1 gene (11), and a remarkable physiological guidance system (1,2) approved for intravenous and hepatic arterial use in humans (9,10,12) is referred to here as Rexin-G (an acronym for the expression vector, its inhibitory function, and therapeutic gene). Rexin-G represents the first in a series of tumor-targeted nanoparticles designed to operate efficiently within the context of the human circulatory system; designed to seek out areas of significant histopathology, while sparing normal non-dividing cells and undamaged tissues; designed to deliver a cell-killing designer gene precisely where it is needed most; designed to target metastatic disease *per se* (2,7,8,13). In 2003, the US FDA granted Orphan Drug Status to Rexin-G, based on critical analysis of safety and efficacy in stage IV pancreatic cancer, followed thereafter by an Expanded Access Designation, specifically for treating all solid tumors that are refractory to standard chemotherapy by the Philippine BFAD in 2006 (10).

With profound demonstrations of single-agent-efficacy in an increasingly broad spectrum of chemo-resistant tumor types, the basic functionality of the enabling platform technologies has been proven beyond academic contestation, and the clinical indications have been validated and expanded appropriately in a series of international clinical trials. Most recently, oncologists in Japan have documented objective responses in a variety of additional cancer types, including lung cancer metastatic to the brain, gastric and bile duct cancer, and soft tissue sarcoma (personal communications). Thus, the increasing clinical applications of Rexin-G may encourage a renewed appreciation of the fundamental principles of biochemistry, enzymology, cancer genetics, virology, functional genomics, molecular engineering, and biopharmaceutical development involved in grasping the 'Sword' of the core biotechnologies as they emerge from the 'Stone' of chemistry and physics. If so, the philosophical tractus entitled 'Nanotechnology Blooms, at last' (14) and the medical tractus entitled 'A Primer on Pathotropic Medicine' for practitioners (15) should serve as practical introductions, if not *vade mecums*. Basically, this newly developed class of disease-seeking nanoparticles incorporate a physiological surveillance function (targeting) derived by an elegant adaptive molecular engineering of von Willebrand (blood coagulation) factor, along with the requisite stealth cloaking devices (membranes), rigid superstructures (capsules), enzymatic activities (programs), and genetic components (therapeutic payload) into a single medicinal entity whose biocompatibility, bioactivity, and bioenergetics are intrinsic to the life sciences, and whose fail-safe designations are naturally enforced. In operational terms, these dutiful nanoparticles seek out and accumulate selectively in metastatic lesions and deliver their tumoricidal designer gene payloads precisely to target cells within the tumor nodules: that is, specifically to cancer cells and the proliferative components of the tumor-associated neovasculature. The resulting tumoricidal pulses enforce apoptosis (programmed cell death), in great masses of tumor cells, which is accompanied and enhanced by profound anti-angiogenesis, or the impairment of blood supply within the tumor nodules.

While MRI, CAT scans, and evaluations of circulating tumor markers are routinely employed to monitor objective clinical responses, as seen during the course of standard cancer therapy, the attainment of post-treatment tissue samples by surgical biopsy is far more fortuitous. However, considering that this kind of therapeutic precision and selective cell destruction was historically all but impossible, such surgical specimens collected during or shortly after treatment with tumor-targeted gene therapy vectors (i.e., Rexin-G) may indeed provide a wealth of new insights into the fundamental mechanisms of tumor destruction, disease stabilization, and tumor eradication that was previously seen only from more remote vantage points in radiologic films. This histological paper represents the first detailed examination and analysis of a limited set of tumor specimens collected following Rexin-G treatment, providing pathologists with a unique and unprecedented opportunity to examine the mechanisms of tumor destruction under the onslaught of the world's first truly-effective targeted injectable genetic medicine for cancer.

The same gene targeting platform embodied in Rexin-G also enables oncologists and cancer immunologists to deliver discrete cytokine genes encoding powerful immune-modulating agents directly to the remote metastatic cancer foci, thus enhancing immune cell recruitment, activation, and maturation, without raising the systemic levels of these powerful cytokines. Moreover, the focal destruction of metastatic tumors can now be combined with the focal recruitment and activation of the patient's immune system by employing a series of different vectors, each carrying different genetic payloads. By enhancing the presentation of an abundance of tumor antigens to the activated immune cells, now present within the same localized compartment, the potential for an effective vaccination may be enhanced significantly without the tedious tumor cell manipulation *ex vivo* needed to generate most cytokine-secreting cancer vaccines to date (16-22).

The second part of this paper is focused on Reximmune-C, a tumor-targeted nanoparticle bearing the full coding sequence for the human GM-CSF (22-25), arrayed as an expression construct, and its clinical utility when used in strategic combination with the exquisitely cytocidal agent, Rexin-G. The aim of the early-stage clinical trials utilizing Reximmune-C, in addition to Rexin-G, is to examine the degree and extent to which these gene therapy approaches would act synergistically, first to facilitate tumor destruction and possibly eradication, and second to recruit a cadre of immune cells whose interactions are needed to provide both humoral and cell-mediated immunity, with the intent on optimizing the sequence and timing of therapeutic agents that could provide a kind of lasting immunity that would prevent or forestall recurrences in the future. This part of the study focuses on the unique histologic and radiologic characteristics of tumors obtained from patients with chemo-resistant tumors who were treated with Rexin-G in combination with Reximmune-C in a personalized vaccination protocol. Here again, this study of the available histological specimens represents a first look at the overt histological and immunocytochemical changes observed

following sequential treatment with this strategic combination of tumor-targeted agents. Taken together, the results of this histopathological study serve to increase our understanding of an emerging biotechnology platform of considerable clinical utility, providing medical oncologists and their prospective patients with a glimpse of things to come.

Materials and methods

Product description (Rexin-G™ and Reximmune-C™). Rexin-G™ and Reximmune-C™ are pathotropic MLV-based nanoparticles or gene delivery vectors, encoding a dominant negative mutant construct of the human cyclin G1 gene or a cytokine gene, GM-CSF, respectively, expressed under the control of a hybrid LTR promoter (10). The vectors also contain a neomycin resistance gene, which is driven by the SV40 early promoter and is utilized for the precise determination of vector titer. The vectors are produced by transient co-transfection of three separate plasmids in 293T cells (ATCC) maintained as a fully validated master cell bank. Producer cell growth medium is comprised of a DMEM base supplemented with 10% γ-irradiated fetal bovine serum (Hyclone). The serum was obtained exclusively from USA sources, and has been tested to be free of bovine viruses in compliance with USDA regulations. The production, suspension, and collection of therapeutic nanoparticles are performed in a final proprietary serum-free formulation, which is then processed by sequential clarification, bioprocessing, filtration, and final fill into cryobags using a sterile closed loop system. The resulting nanoparticles, with an average diameter of 100 nanometers, are devoid of all viral genes, and are free of replication competent retrovirus (RCR) based on the FDA-CBER Points to Consider testing guidelines. The titers of the clinical lots range from $2x10^7$ (Reximmune-C) to $5x10^9$ (Rexin-G) colony-forming units (CFU)/ml, and each lot is validated for requisite purity and biological potency.

Shipping, storage, preparation, and infusion of Rexin-G and Reximmune-C. Processed clinical-grade materials in sealed cryobags are stored in a -70±10˚C freezer prior to shipment. The validated and released vector bags are shipped on dry ice to the clinical site where the vector bags are stored in a -70±10˚C freezer until used (9,10). Fifteen minutes before intravenous infusion, the vector in the cryobag is rapidly thawed in a 35-37˚C water bath and immediately infused via a peripheral vein or a central IV line. Each bag of Rexin-G or Reximmune-C is infused over 10-30 min at a rate of 4 ml/min.

Clinical protocols. After formal informed consent was obtained, eligible patients participated in an expanded access protocol or a phase I/II protocol approved by the Philippine Bureau of Food and Drugs, and the Institutional Review Board or Hospital Ethics Committee. The clinical studies evaluated the cumulative safety/toxicity and the efficacy of intravenous infusions of Rexin-G or the safety and potential efficacy of Rexin-G plus Reximmune-C, in patients with solid tumors considered refractory to standard chemotherapy (10). Tumor specimens were made available for studies from resected or biopsied metastatic tumors from six patients as part of standard care practice, and from one patient post-mortem.

Harvest of tumors and immunohistochemical staining of tissue sections. The tumors were fixed in 10% formalin prior to processing. Tissue sections were stained with hematoxylin-eosin stain, trichrome stain for detection of collagenous proteins, or immunochemically-stained for presence of TUNEL-positive apoptotic cells, and tumor infiltrating lymphocytes (TILs) including $CD4^+$ (T_h), $CD8^+$ (T_c), $CD68^+$ (macrophage), $CD138^+$ (plasma B cell), $CD35^+$ (dendritic), $CD20^+$ (B cell), and $CD45^+$ (monocyte-macrophage) cells. Appropriate tissue sections were immunochemically-stained for presence of $GM\text{-}CSF^+$ cells to document GM-CSF transgene expression and paracrine secretion by *in vivo*-transduced tumor cells, or for presence of CA15.3 $antigen^+$, Melan A^+, or $EGF\text{-}R^+$ cells to document the identity of the tumor cell in certain patients with adenocarcinoma of breast, malignant melanoma, and non-small cell lung cancer (NSCLC), respectively.

Results

Initial preclinical proofs of concept for tumor-targeting and cellular transduction with pathotropic nanoparticles in a mouse model of metastatic cancer. The diagram shown in Fig. 1(I), illustrates the method of establishment of a murine model of liver metastasis. The remarkable efficacy of pathotropic nanoparticles in targeting metastatic cancer cells *in vivo* in this nude mouse model of liver metastasis was tested after infusion of $7x10^5$ human pancreatic cancer (MiaPaca2) cells into the portal vein via an indwelling catheter that was kept in place for 5 days (7). A single infusion was given three days later, using a pathotropic-targeted or non-targeted vector bearing a nuclear ß-galactosidase reporter gene. The mice were sacrificed two days later on day 5. Histologic and histochemical evaluations of metastatic tumor foci from mice treated with a targeted or non-targeted ß-galactosidase vector were performed and evaluated with an Optimas imaging system.

As shown in Fig. 1(I)A-C, the earliest stages of tumor invasion are recognized by the targeted nanoparticles: enhanced transduction of metastatic tumor cells (>50%) in the hepatic tumor nodules at or near disrupted hepatic venules (arrows) which are areas of tumor entry into the liver parenchyma from the circulatory system. Well established tumors with active neoangiogenesis are also targeted, as Fig. 1(I)D-F, show significant transduction of actively proliferating tumor-associated stromal cells and vascular endothelial cells (indicated by arrows) in tumor foci undergoing angiogenesis, but not in neighboring non-dividing normal hepatocytes. No transduction was observed in metastatic foci using non-targeted control vectors (data not shown). In these preclinical studies, there was no evidence of hepatocyte injury, vessel thrombosis, or cholestatic changes in tissue sections from vector-treated animals. We conclude that pathotropic nanoparticles are efficiently targeted to tumor cells and tumor-associated, proliferating stromal and vascular cells without uptake or integration into normal, non-dividing liver cells.

GM-CSF production and cytokine-directed recruitment of host mononuclear cells in established tumor xenografts in mice treated with Reximmune-C. Fig. 1(II) illustrates the establishment of subcutaneous tumor xenografts in athymic

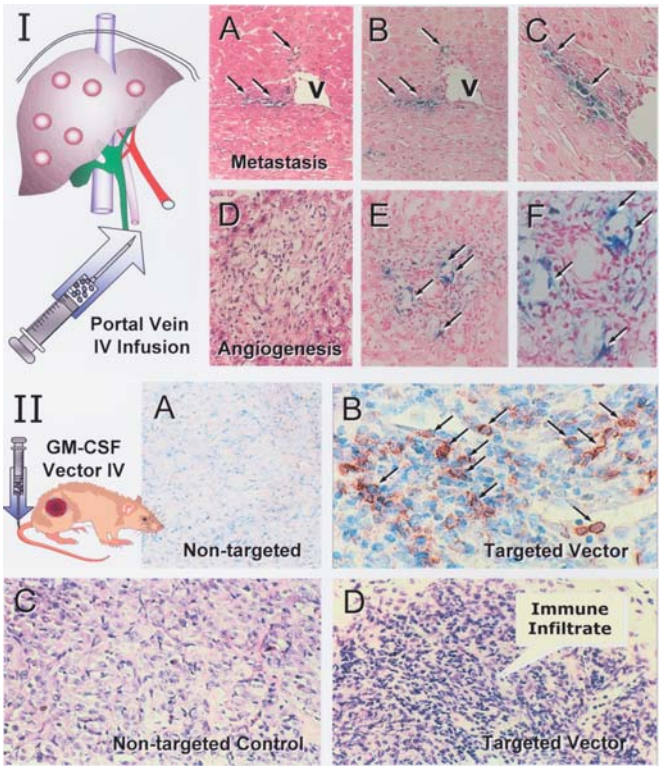

Figure 1. Preclinical studies of pathotropic tumor targeting in murine models. (I), *In vivo* gene transfer studies in a nude mouse model of liver metastasis. The efficiency of pathotropic tumor targeting *in vivo* was examined after infusion of $7x10^5$ human pancreatic cancer (MiaPaca2) cells into the portal veins of nude mice via an indwelling catheter. Vector infusions were started three days later, using a targeted or non-targeted control vector, each bearing a nuclear ß-galactosidase reporter gene. Two days later, the mice were sacrificed and histologic, histochemical and immunohistochemical evaluations of metastatic tumor foci from mice treated with targeted or non-targeted vectors of equivalent titers were performed. (A), Hematoxylin and eosin (H&E)-stained tissue sections of liver from a ß-galactosidase vector-treated mouse with experimental liver metastasis; arrows point to early-stage tumor cell invasion from a hepatic venule (v). (B), X-Gal-stained tissue section of (A) counterstained with nuclear fast red stain, where selective gene transfer (arrows) is indicated by blue-green stain. (C), Higher magnification of (B) where arrows identify ß-galactosidase - expressing tumor cells. (D), H&E-stained tissue section from a targeted ß-galactosidase vector-treated mouse exhibiting a well-formed tumor nodule with active angiogenesis. (E), X-Gal-stained liver sections counterstained with nuclear fast red stain reveal significant transduction of the proliferative vascular cells (arrows). (F), Higher magnification of (E) confirms ß-galactosidase expression in endothelial cells (arrows), in addition to tumor cells. (II), Targeted GM-CSF gene transfer and cytokine-directed recruitment of host mononuclear cells in established tumor xenografts. Subcutaneous tumor xenografts were established in athymic nu/nu mice by subcutaneous implantation of $1x10^7$ MiaPaca2 cells. When the tumors reached a size of ~20 mm^3, 200 μl of either the targeted GM-CSF vector (i.e., Reximmune-C) or a non-targeted GM-CSF vector were injected directly into the tail veins each day for 10 days (cumulative vector dose: $2x10^7$ CFU for each vector). The mice were sacrificed one day after completion of the treatment, and the harvested tumor sections were immunostained for presence of the GM-CSF transgene, using a goat polyclonal anti-GM-CSF antibody (N19). In contrast to the non-targeted vector, where little if any transgene expression (<1%) was evident (A), abundant immunoreactive GM-CSF protein was observed (arrows) in a significant proportion (~35%) of tumor cells throughout the tumor nodules of Reximmune-C vector-treated mice (B). (C), H&E section of a tumor nodule from a control animal showing robust tumor formation with little evidence of immune infiltrate, compared with the GM-CSF-secreting tumors of targeted vector treated mice (D), where significant recruitment and infiltration of host mononuclear cells is observed.

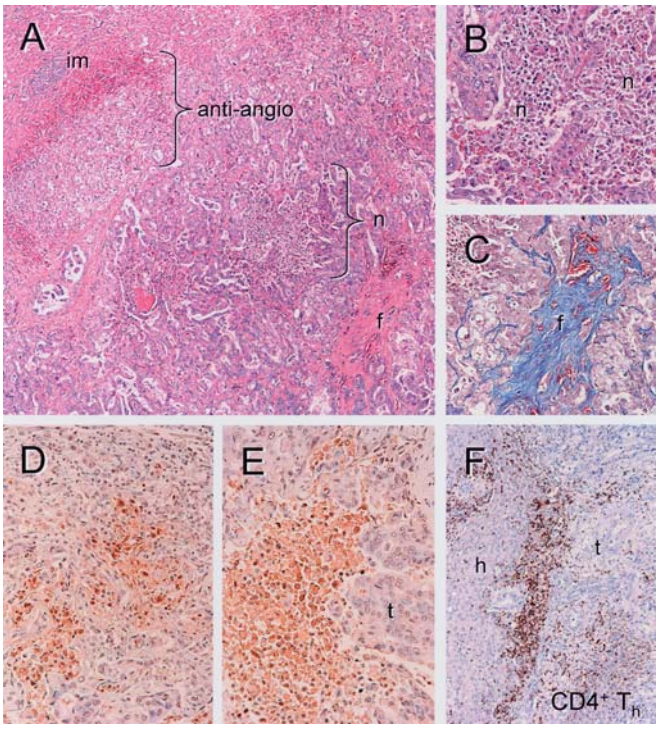

Figure 2. Liver biopsy - intravenous Rexin-G treatment induces apoptosis, necrosis, fibrosis, and focal disruption of tumor vasculature within metastatic nodules in a patient with pancreatic cancer metastatic to liver. (A), Representative H&E-stained tissue section of a tumor nodule within a liver biopsy showing extensive necrosis, fibrosis and disrupted blood supply with limited bleeding within the tumor; t, tumor cells; n, necrosis; f, fibrosis; im, mononuclear cell (immune) infiltrates; anti-angio, disrupted tumor vasculature. (B), Higher magnification of the necrotic formations seen in (A). (C), Trichrome stain of a serial section of same tumor nodule. Blue-staining material indicates presence of collagenous proteins thereby highlighting reactive fibrosis. (D and E), TUNEL staining for apoptotic structures (brown-staining material) reveals programmed cell death as a major feature in both diffuse areas (D) and focal areas (E) of tumor cell death. (F), Immunostaining for $CD4^+$ antigens identifies a significant complement of helper T cells (T_h) in the immune infiltrate, primarily located near the boundaries between hepatic (h) and tumorous (t) tissues (A).

nu/nu mice by subcutaneous implantation of $1x10^7$ MiaPaca2 cells (8). When the tumors reached a size of ~20 mm^3, 200 μl of either the targeted vector (Reximmune-C) or CAE-GM-CSF non-targeted control vector, was infused into the tail vein daily for 9 days (cumulative vector dose: $2x10^7$ CFU for each vector). The mice were sacrificed one day after completion of treatment, and the harvested tumor sections were immunostained for presence of GM-CSF transgene, using a goat polyclonal anti-GM-CSF antibody (N19). The targeting of a remote metastatic tumor nodule following intravenous infusion is demonstrated by subsequent immunohistochemistry. Fig. 1(II)B, reveals the expression of immunoreactive GM-CSF protein in a large proportion (~35%) of tumor cells throughout the tumor nodules of Reximmune-C vector-treated animals, while negligible transgene expression is seen in the non-targeted CAE-GM-CSF vector-treated controls Fig. 1(II)A. Further, H&E sections of tumor nodule shows the dramatic recruitment of host mononuclear cells into the tumor nodules after repeated intravenous injections of Reximmune-C in tumor-bearing mice Fig. 1(II)D; in contrast, no such recruitment of host mononuclear cells was noted when using the non-targeted control vector Fig. 1(II)C, where little if any immune infiltrate is observed.

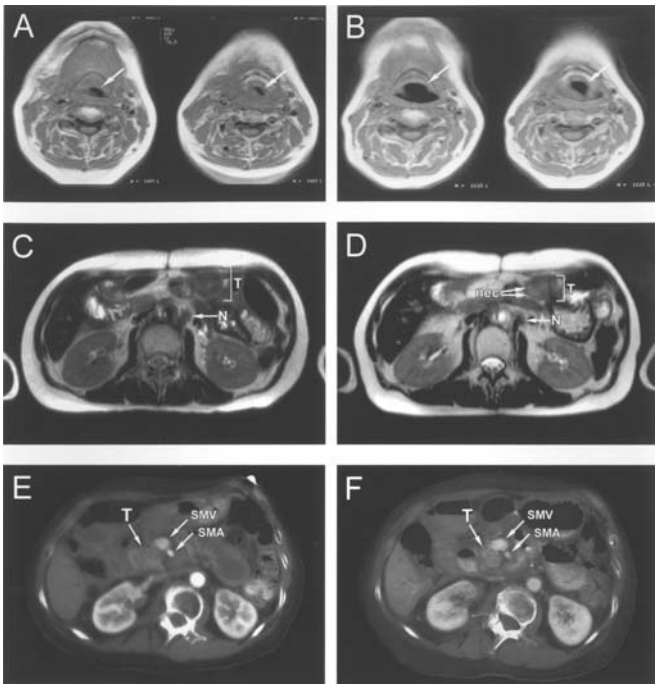

Figure 3. Composite imaging studies before and after Rexin-G treatment demonstrates tumor regression in patients with chemo-resistant solid tumors. MRI images of the neck region obtained from a patient with squamous cell carcinoma of the larynx before (A), and after (B) Rexin-G treatment. Measurement of the diameters of serial sections of the upper airway shows a ~300% increase in the upper airway diameters (white arrows) after repeated infusions of Rexin-G when compared to sections obtained prior to treatment. The increased patency of the airway corresponded to a profound reduction of the surrounding tumor masses, and a gradual return of vocal capabilities. (C), MRI images of the abdomen obtained from a patient with metastatic pancreatic cancer, one day after completion of a treatment cycle showing a large round recurrent tumor (T, bracketed area) in the region of the pancreas within the area of the surgical bed and an enlarged para-aortic lymph node (N) indicating metastasis. Follow-up MRI from the same patient after completion of a second treatment cycle (D) showing an irregularity in the shape of the recurrent tumor (T, bracketed area) with a large area of central necrosis (nec) involving 40-50% of the tumor mass, and a significant decrease in the size of the para-aortic lymph node metastasis (N). (E), Representative abdominal CT scan from a patient with locally advanced pancreatic cancer before Rexin-G treatment revealing a 6.0-cm^3 mass in the region of the pancreatic head (T) encroaching on the superior mesenteric vein (SMV) and the superior mesenteric artery (SMA). Follow-up abdominal CT scan from same patient after Rexin-G treatment (F) revealing that the pancreatic tumor mass (T) has decreased in size by 47% and has regressed away from the superior mesenteric vessels (SMV and SMA).

Intravenous infusions of Rexin-G induces apoptosis, necrosis, fibrosis, and disruption of tumor vasculature in the tumor nodules of a patient with stage IV pancreatic cancer metastatic to the liver. A histological analysis of this particular patient, in whom 6 of 8 liver tumor nodules disappeared entirely (confirmed by CT scan) after repeated Rexin-G infusions (cumulative dose: $5x10^{11}$ CFU), was facilitated by means of a liver biopsy (Fig. 2). Examination of the [battle]field under low magnification (Fig. 2A) reveals a series of discrete, yet interrelated, activities, including focal areas of overt anti-angiogenesis associated with degenerating tumor cells (indicated by brackets), as well as large areas of necrosis (n) and reactive fibrosis (f). Closer examination of the necrotic (Fig. 2B) and fibrotic (Fig. 2C) zones provide additional cellular detail, while TUNEL stains for apoptotic structures confirm that apoptosis (programmed cell death) is indeed a major mechanism of tumor cell death in both generalized (Fig. 2D) and focal areas (Fig. 2E). Consistent with radiological observations of central necrosis in metastatic tumor nodules following Rexin-G treatment, these histological observations of apoptosis, necrosis, and fibrosis within the tumor nodules (Fig. 2A-E) are remarkably similar to those observed in preclinical studies (7,8), along with the observations of tumor infiltrating lymphocytes, as seen in the residual liver tumors of this biopsied liver (Fig. 2F, $CD4^+$ stain). The appearance of immunoreactive T and B lymphocytes (data not shown) infiltrating from the normal hepatic tissues (h) into the areas of tumor (t) destruction in the residual liver tumors indicates that Rexin-G does not itself suppress or preclude local reactive immune responses.

Regression of primary and metastatic tumors after Rexin-G infusions in one patient with squamous cell carcinoma of the larynx, and two patients with locally-advanced and metastatic pancreatic cancer. Fig. 3A and B show MRI images of the neck of a patient with squamous cell carcinoma of the larynx before and after Rexin-G treatment (cumulative dose: $2x10^{11}$ CFU). Radiographic measurement of the diameters of serial sections of the upper airway showed a dramatic (~300%) increase in the upper airway diameters after repeated infusions of Rexin-G when compared to sections obtained prior to treatment (indicated by white arrows). The increased patency of the airway corresponded to a profound reduction of the surrounding tumor masses and, remarkably, a gradual return of vocal capabilities.

Fig. 3C and D show abdominal MRI images taken from a patient with widely metastatic pancreatic cancer before and after Rexin-G infusions (cumulative dose: $2.1x10^{11}$ CFU). Follow-up abdominal MRI revealed: i) no new areas of tumor metastasis, ii) discernable areas of central necrosis, involving 40-50% of the primary tumor (T), and iii) a significant decrease in the size of the para-aortic abdominal lymph node (N) following treatment (Fig. 3D) compared to baseline studies (Fig. 3C).

Fig. 3E and F show abdominal CT images taken from a patient with locally advanced pancreatic cancer before and after Rexin-G treatment (cumulative dose: $1.8x10^{11}$ CFU). Fig. 3E reveals a 6.0-cm^3 mass in the region of the pancreatic head (T) encroaching on the superior mesenteric vein (SMV) and the superior mesenteric artery (SMA). In contrast, a follow-up abdominal CT scan after Rexin-G treatment shows that the primary tumor had decreased in size by 47% (Fig. 3F) and that the pancreatic tumor mass (T) had regressed away from the superior mesenteric vessels (SMV and SMA), raising the potential for a curative surgical resection.

Intravenous Rexin-G induces varying degrees of necrosis, apoptosis, fibrosis and infiltration by antigen presenting cells (APCs) in a metastatic inguinal lymph node obtained from a patient with malignant melanoma. Examination of a large field of the biopsied inguinal lymph node (Fig. 4A) in a patient undergoing Rexin-G treatment for malignant melanoma (cumulative dose: $5.6x10^{11}$ CFU) reveals large areas of necrosis (n), influenced by anti-angiogenic activities (bracket), fibrotic reactions (f), and significant immune infiltrate (im) within in the context of flagrant tumor (t) growth. Distinctive zones of hemosiderin-laden macrophages are observed (Fig. 4A box,

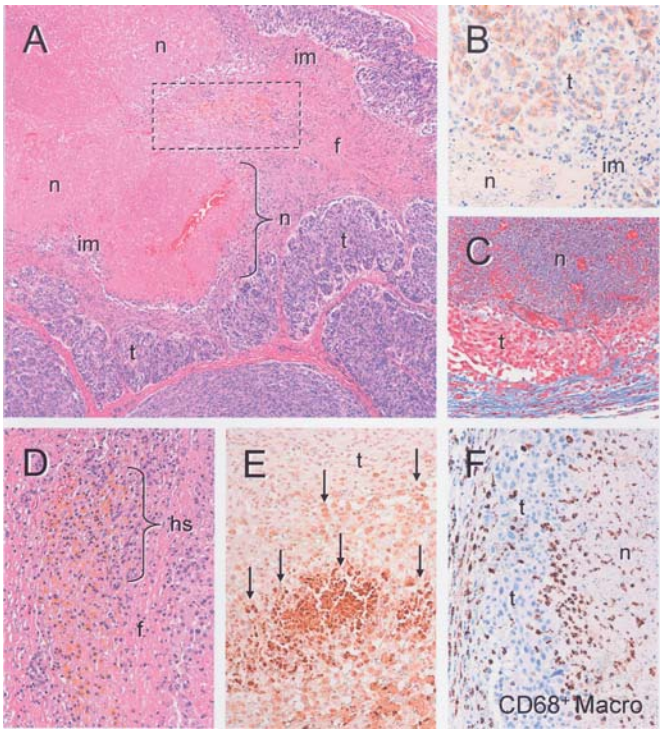

Figure 4. Intravenous Rexin-G induces apoptosis, necrosis, anti-angiogenesis, and fibrosis, with significant infiltration of antigen presenting cells (APCs) in a metastatic inguinal lymph node obtained from a patient with malignant melanoma. (A), H&E-stained tissue sections of inguinal lymph node revealing extensive necrosis (n), fibrosis (f) and mononuclear cell infiltration (im) with viable tumor cells (t) in the periphery. (B), Immunostaining for Melan A (melanin) confirms the cellular derivation of the metastatic tumor (t) as a malignant melanoma; areas of necrosis (n) and mononuclear cell infiltrates (im). (C), Trichrome staining of tissue sections is used to highlight collagenous extracellular matrix proteins (blue-staining material) prominent in areas of necrosis (n) and angiogenesis, as well as in areas of flagrant tumor (t). (D), Higher magnification of (A, boxed) revealing golden-yellow hemosiderin-laden macrophages resolving a localized [battle]field of blood elements and tumor cell debris. (E), TUNEL staining identifies focal masses of apoptotic cells (indicated by reddish-brown staining material) within the metastatic lymph node (indicated by arrows). (F), Immunostaining for the CD68 antigen identifies antigen-presenting CD68$^+$ macrophages infiltrating tumorous (t) and necrotic areas (n) within the lymph node.

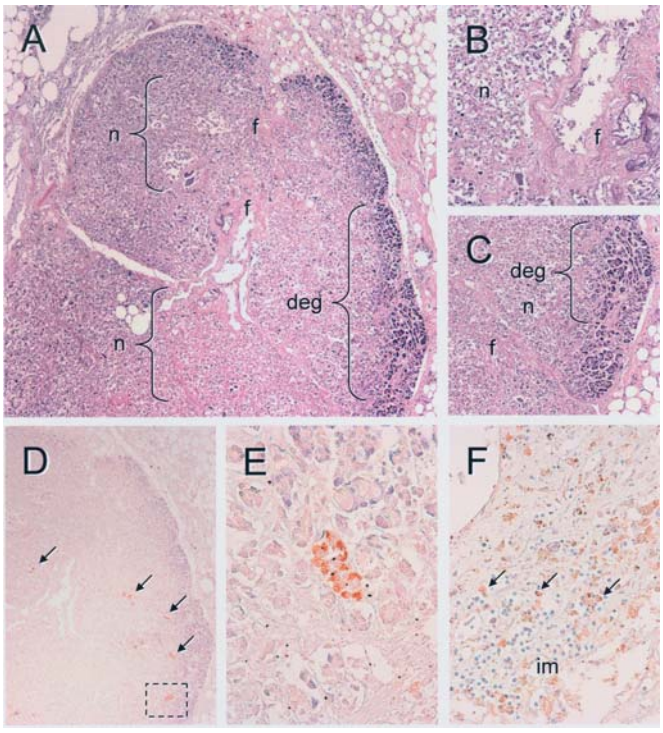

Figure 5. Sequential intravenous infusions of Rexin-G plus Reximmune-C produces extensive necrosis and confirms local GM-CSF production within the primary pancreatic tumor of a patient with intractable stage IV pancreatic cancer. (A), H&E-stained tissue sections of primary pancreatic tumor demonstrate extensive (~95%) necrosis (n) of cancer cells, with some reactive fibrosis (f), with a degenerative (deg) rim of viable tumor cells and organoid structures seen at the periphery. (B and C), Higher magnification of the fibrotic, necrotic, and degenerative areas of the section seen in (A). (D), Immunostaining for the GM-CSF transgene identifies small clusters of immunoreactive GM-CSF secreting tumor cells (arrows) remaining within this inoperable primary tumor. (E), Higher magnification of (D) showing immunoreactive GM-CSF protein within viable residual tumor cells (indicated by reddish-brown staining material). (F), Close examination of areas with significant immune infiltrate, are indicative of GM-CSF positivity in necrotic tumor cells (indicated by arrows) and in fragments of tumor cell debris accompanied by mononuclear cell infiltration (im).

enlarged in D), and the major mechanism of cell death - within this sentinal lymph node - by apoptosis *en mass* is verified by TUNEL stain (Fig. 4E). The identification of the metastatic tumor type as malignant melanoma was confirmed by the presence of melanin pigment in the tumor cells (t) themselves (Fig. 4B). Trichrome stain for extracellular matrix proteins (bright blue) provides a pathotropic-eye view of the stromal, vascular, and necrotic tumor formations (Fig. 4C). This striking revelation that the circulating pathotropic nanoparticles have not only transited through the human circulatory system, but have penetrated and accumulated in the diseased lymphatic nodes to an extent that the cytocidal gene delivery system is still seen to be working to eradicate the cancerous elements has remarkable implications. These findings indicate that the pathotropic nanoparticles in Rexin-G retain their bioactivity as they circulate throughout the body, not only accumulating in vascularized primary and metastatic lesions but also draining into the lymphatic system with therapeutic impact. Further, the preponderance of CD68$^+$ macrophages (antigen-presenting cells; Fig. 4F) surrounding viable tumor nodules, and to a lesser extent, CD4$^+$ helper T cells and CD8$^+$ killer T cells (data not shown) affirm the potential of using pathotropic nanoparticles bearing cytokine genes for *in situ* cancer vaccination strategies.

Massive necrosis of primary tumor and evidence of GM-CSF secretion in a patient with advanced metastatic pancreatic cancer treated with Rexin-G followed by Reximmune-C. When radiation and chemotherapy fail to control the spread of metastatic pancreatic cancer to and in the liver, the tumor burden within this vital organ can grow to enormous proportions, displacing normal liver parenchyma with massive tumor formations. At such times, compassionate use and informed consent combine to encourage the application of more aggressive protocols to reduce the lethal tumor burden. Fig. 5 shows a series of sections showing extensive necrosis of the primary tumor in an autopsied tumor specimen obtained from a patient with intractable metastatic pancreatic cancer that was treated with successive infusions of Rexin-G for 28 days (cumulative dose: $2x10^{12}$ CFU) followed by Reximmune-C for 6 days (cumulative dose: $3x10^{10}$ CFU). While the series of infusions were well-tolerated, and the overall tumor burden was reduced significantly, the patient failed to thrive and to

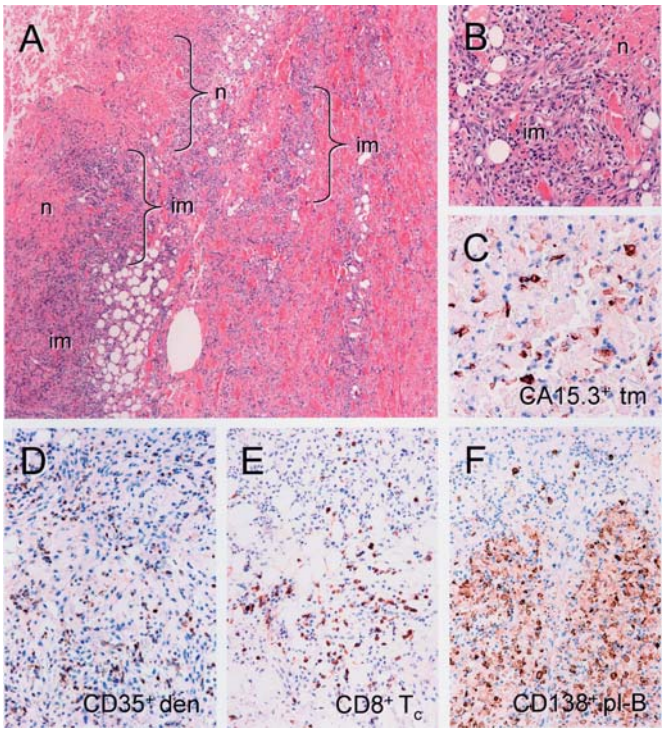

Figure 6. Surgical biopsy from a patient with adenocarcinoma of the breast following Rexin-G plus Reximmune-C treatment demonstrates near-eradication of the tumor cells with evidence of active immunization within the metastatic tumor nodule. (A), H&E-stained tissue sections of a post-treatment tumor nodule revealing extensive necrosis (n), and mononuclear immune cell infiltration (im) along with stromal elements within the residual tumor, which now lacks discernable formations of flagrant tumor cells. (B), Higher magnification of sections shown in (A) is confirmatory. (C), Immunostaining for the tumor marker CA15.3 helps to identify small numbers of remaining CA15.3$^+$ breast cancer cells among the dead and dying tumor cells. (D, E and F), Detailed immunocytochemical studies identify a multitude of CD35$^+$ dendritic cells (D), CD8$^+$ killer T cells (E) and CD138$^+$ plasma B cells (F), with lesser amounts of CD68$^+$ macrophages and CD20$^+$ B cells (not shown), suggesting a relatively mature or advanced immune response. Note, the professional antigen-presenting dendritic cells and the antibody-manufacturing plasma cells are derived from less mature monocytes and B lymphocytes, respectively, following their activation by antigens. These observations provide strong support for the concept of personalized vaccination *in situ*, facilitated by the presence of tumor neo-antigens and activated immune cells in the same locale.

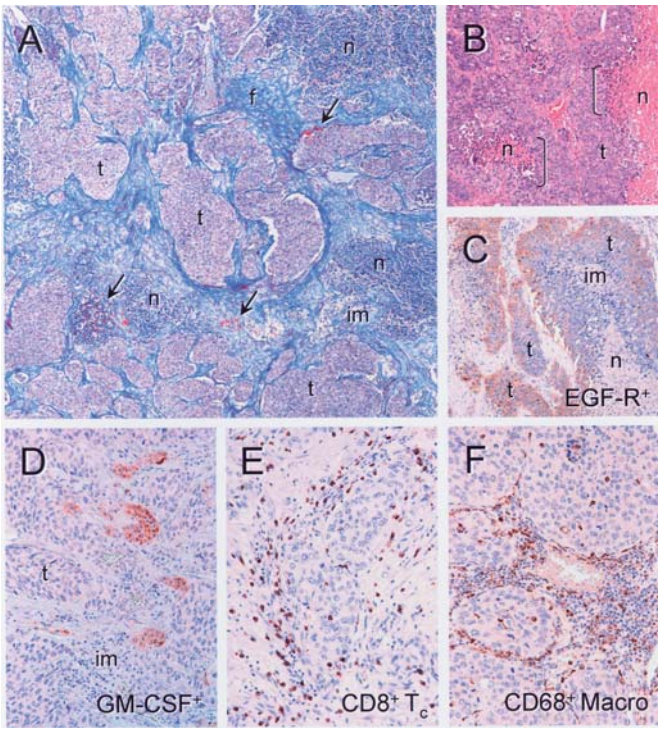

Figure 7. Surgical specimen from a patient with non-small cell lung cancer (NSCLC) metastatic to adrenal glands provides a unique view of the histological [battle]field within this afflicted organ, revealing the potential for a well-disposed immunization. (A), Trichrome-stain for collagenous proteins (bright blue) reveals a pathotropic-eye view of an aggressive metastatic cancer, as seen in these invasive and very extensive metastatic tumor formations that have eroded and displaced the normal histology of the adrenal gland. Following treatment with Rexin-G and Reximmune C, we find large areas of necrosis (n) and micro-hemorrhages indicative of anti-angiogenesis (arrows), accompanied by significant immune infiltrate (im) and reactive fibrosis (f), as indicated by blue-staining material, and relegating the tumor to veritable islands-under-siege. (B), A more-standard H&E stain is provided for comparison. (C), Immunostaining for EGF-R expression in the tumor nodules confirms the NSLC cell origin, yet indicates a phenotypic variability which suggest that some but not all of the remaining tumor cells actually over-express the EGF receptor; overt apoptosis, focal necrosis (n), and significant immune infiltration into the islands of tumor cells (t) is also evident here. (D), Transgene expression following Reximmune C is confirmed by immuno-staining, as GM-CSF immunoreactivity (red-brown staining material) was noted in a small but appreciable percentage of tumor cells (t) near an area of immune cell infiltration (im) thus demonstrating successful targeting of the cytokine transgene expression. (E and F), Evaluation of the recruited complement of TILs by selective immunostaining showed significant populations of both immunoreactive CD8$^+$ killer T cells (E) and CD68$^+$ macrophages (F), with lesser amounts of CD35$^+$ dendritic cells, CD138$^+$ plasma cells, CD4$^+$ T helper cells, and CD56$^+$ natural killer cells; this may be indicative of a less mature but no less important host immune response than that seen in the metastatic tumor nodule in Fig. 6.

readily resolve the large lesions, necessitating supportive care. Unfortunately, the patient died of a fulminant *Escherichia coli* bacterial sepsis three months after treatment, which was considered to be unrelated to the Rexin-G intervention, yet may relate to the problem of post-ablative wound healing in a more general sense. However, histological examination of the extent of the tumor destruction is informative. As seen in Fig. 5A, and enlarged in Fig. 5B and C, post-mortem findings indicate a massive amount of necrosis (n) involving ~95% of this pancreatic tumor with various areas of fibrosis (f), flanked by degenerative (deg) and organoid structures. Immunohistochemical staining for GM-CSF identified several areas where tumor cells expressing GM-CSF (Fig. 5E and F) were evident (arrows) in small islands (boxed area, enlarged in Fig. 5E), and significant immune infiltrate (im) is seen in the vicinity of what appears to be necrotic fragments of GM-CSF secreting cells (Fig. 5F). This clinical case study highlights three important issues: i) the overall importance of treating patients earlier, before cancer produces irreparable organ damage, ii) the potential for Rexin-G to meet and match extremely large tumor burdens, and iii) the potential for Reximmune-C, with its immune-stimulating payload, to participate in the process of tumor destruction.

Complete eradication of metastatic tumor nodule and evidence of active immunization in a patient with adenocarcinoma of the breast following treatment with successive infusions of Rexin-G, Reximmune-C and Rexin-G (Tri-Rex protocol). Viewed under low magnification, this tumor biopsy (Fig. 6A) illustrates a fully necrotic tumor nodule with extensive areas

of necrosis (n) and significant infiltrations of host mononuclear cells (im) with little if any flagrant tumor cells remaining after successive infusions of Rexin-G (cumulative dose: $6x10^{11}$ CFU), followed by Reximmune-C (cumulative dose: $1x10^{10}$ CFU) followed by Rexin-G (cumulative dose: $4x10^{11}$ CFU) in a metastatic tumor specimen obtained from a patient with breast cancer with widespread metastasis to lymph nodes, liver, lung and bone. An enlargement of the dying/dead tumor nodule (Fig. 6B) confirms the preponderance of necrotic tumor cells (n) and viable immune cells (im) to the extent that it required immunochemical staining techniques to identify the few remaining dead and dying tumor cells by their characteristic CA15.3 breast cancer marker (Fig. 6C). Further characterization of the immune infiltrate revealed an extensive complement of $CD35^+$ dendritic cells (Fig. 6D), $CD8^+$ killer T cells (Fig. 6E) and $CD138^+$ plasma B cells (Fig. 6F). Taken together with the degree of tumor cell eradication documented, these findings provide the first evidence of active immunization occurring *in situ* within the metastatic tumor nodule itself. The presence (i.e., migration, activation and presumptive conversion) of a relatively mature cadre of TILs that function in the context of cell-mediated and humoral immunity, suggests the potential for cancer immunization in an immune competent host.

Pathotropic view of metastatic disease, emphasizing the potential for remote tumor targeting and suggesting the possibility of auto-immunization, in a patient with NSCLC metastatic to the adrenal glands. Surveying the histological [battle]field in which aggressive metastatic lung cancer cells have entirely obliterated the delicately stratified morphology of the adrenal gland (Fig. 7), we find the now-familiar, almost characteristic hallmarks of invasive cancer, as seen from the point of view of a pathrotropic nanoparticle that seeks out and destroys diseased and damaged tissues. As revealed by the trichrome stain (Fig. 7A), an abundance of brilliant blue ECM proteins are exposed by the disruptive biochemical activities of tumor invasion, angiogenesis, and reactive fibrosis; while additional ECM proteins are being exposed by the equally aggressive activities of Rexin-G - anti-angiogenesis (arrows), focal necrosis (n), reparative fibrosis (f) - as well as the enhanced immune responses (im) engendered by Reximmune-C, which together have relegated the metastatic cancer to a series of degenerative islands surrounded by a rising tide of pathotropic impact. The enhanced effective local concentration of pathotropic nanoparticles within the metastatic tumors has facilitated the effective delivery of the cytocidal gene present in Rexin-G that compelled the cells to self-destruct, resulting in visible apoptosis and necrosis throughout the remaining tumors (Fig. 7B, std. H&E stain). The nature of the invading tumor cells were confirmed by immunostaining for overexpression of the EGF-receptor, which is seen in some, but not all, of the NSLC-derived tumor formations (Fig. 7C); tumor formations (t) that each exhibit the characteristics of apoptosis, immune infiltrate (im) and progressive necrosis (n). Moreover, the targeted delivery of the GM-CSF gene into tumor cells, as enforced by the strategic use of Reximmune-C, is verified by the presence of immunoreactive $GM\text{-}CSF^+$ (brown-staining material) within a number of remaining tumor cells, that are apparently attracting immune cells (Fig. 7D). Active immunization is suggested by a vast number of $CD8^+$ killer T cells (Fig. 7E) and $CD68^+$ macrophages (Fig. 7F) that represent the major antigen presenting cells or APCs surrounding these areas of viable tumor and apoptotic cells. However, the proportions of macrophage vs. dendritic cells, and plasma cells vs. B cells suggests that this immune response may be somewhat less mature than that seen in Fig. 6. These findings provide further support for the concept of a personalized vaccination strategy, presenting tumor neo-antigens *in situ* to the activated immune system, not only to assist in the eradication of the tumor, but to provide an additional opportunity for the patient to mount a lasting anti-tumor immunity.

Discussion

The ability of strategically engineered, pathotropically targeted, biologically compatible nanoparticles to seek out and accumulate in diseased tissues - specifically metastatic tumors - and to deliver therapeutic genes within these lesions to such an extent as to alter the course of the disease process is by now a *fait accompli*. With every major biotechnological advance, and every new clinical validation, comes the additional imperative for careful examination, critical analysis, and refinement of both the instruments and the applications of the technology. As the world's first targeted injectable vector continues to reduce intractable tumor burdens in the clinic, extending lives and enabling additional treatment options (10), there is a corresponding call for more refined histopathological analysis and more appropriate clinical endpoints (15) to be developed. Indeed, the enabling targeting technologies embodied in Rexin-G may one day enable the tumor targeting of diagnostic reporter genes with unprecedented digital precision and high-resolution that will revolutionize the field of image analysis (26-28); however, the first generation of pathotropic diagnostic constructs are in the final stages of development (at Epeius Biotechnologies) and have yet to reach the clinic. Meanwhile, further understanding the disease processes, as well as the intended mechanisms of action of the prospective interventional agents are crucial in establishing and interpreting the clinical endpoints that are appropriate for pathotropic medicines at the various stages of treatment: including induction, intensification, maintenance and/or vaccination. To this end, we studied the characteristic histopathologic features of tumors obtained from patients who received either Rexin-G as monotherapy, or Rexin-G (cytoablative) in combination with Reximmune-C (immune-stimulating) whenever tumor tissue was made available as a part of standard care, after diagnostic biopsy, surgical resection, or autopsy.

In an extensive series of preclinical studies of safety, efficiency, and efficacy, we previously showed that pathotropic nanoparticles can effectively deliver marker genes to metastatic foci with remarkable penetrance - e.g., when injected into the portal vein in a nude mouse model of liver metastasis [Fig. 1(I)] - and that intravenous infusions of the cytocidal agent Rexin-G induces profound apoptosis, necrosis, and compensatory fibrosis in remote tumor xenografts when infused intravenously through the tail veins of tumor-bearing mice (8). Extrapolation of the preclinical dose-response

phenomena enabled direct translation from the bench to the bedside with remarkable consistency of clinical effect (9,10). In subsequent preclinical studies, we demonstrated that repeated intravenous infusions of Reximmune-C (GM-CSF transgene) in the murine model resulted in significant transduction of tumor xenograft cells, prompting a local paracrine secretion of immunoreactive GM-CSF protein [Fig. 1(I-II)B] within the metastatic tumors without raising the systemic blood levels of this powerful immune-modulating cytokine. Furthermore, the localized expression of the GM-CSF transgene - even in nude mice, which lack T cell functions - promoted the recruitment of a significant complement of host mononuclear cells into the tumor xenografts [Fig. 1(II)D], a complement of TILs that consisted primarily of $CD86^+$ dendritic cells and $CD40^+$ B cells. These findings, taken together with the clinical data, attest to the tumor-targeting and gene delivery capabilities of these pathotropic nanoparticles, and perhaps their capabilities to revolutionize the fields of cancer gene therapy (29) and cancer immunotherapy (30,31).

In agreement with the results of these vanguard preclinical studies, significant apoptosis, necrosis, and reparative fibrosis - along with varying degrees of hemorrhagic necrosis and overt anti-angiogeneis - were consistently observed in tumors obtained from both Rexin-G and from Rexin-G plus Reximmune-C-treated patients (Figs. 2, 4-7). A striking observation is the conspicuous and extensive penetrance of Rexin-G, not only into primary tumors (Fig. 5) and metastatic foci (Figs. 2, 6 and 7), but also into lymph nodes (Fig. 4), inducing tumor regression in both regional and distant lymph nodes, as viewed by MRI imaging (Fig. 3C and D). For example, telltale patterns of apoptosis and necrosis of tumor cells are readily observed in the inguinal nodes, presumably the sentinel lymph nodes, in a patient with malignant melanoma (Fig. 4A, C and E). Since the lymphatic system is a major route of metastasis for many types of cancer, the clinical significance of the finding that Rexin-G is able to travel through the lymphatic system as well as the circulatory system, to remain biologically active during this remarkable journey, and to induce apoptosis and necrosis in sentinel lymph nodes and distant lymph nodes wherein the immune cells are naturally located, cannot be overstated. In tentative support of this conclusion, the patient with metastatic melanoma with lymphatic involvement (Fig. 4), who was treated with Rexin-G as monotherapy, is still alive and enjoys a sustained clinical remission for over 14 months from the start of Rexin-G treatment.

The tumors of patients treated with Rexin-G plus Reximmune-C appeared to have a greater degree of necrosis, hemorrhage, and cystic formations than the tumors of patients treated with Rexin-G alone. In one patient with widespread metastatic breast cancer (Fig. 6), the biopsied tumor nodule showed complete necrosis with no residual tumor, leaving only its footprints, i.e., traces of $CA15.3^+$ antigen amongst dying cells in the necrotic areas (Fig. 6C), while focal areas of persistent immunoreactive $GM\text{-}CSF^+$ cells were seen in tumors from two patients who received Rexin-G plus Reximmune-C. Another distinctive feature of tumors obtained from Rexin-G plus Reximmune-C-treated patients is the abundance of tumor-infiltrating lymphocytes within the dying tumor wherein the person's autoantigens are newly exposed. These include the respective complements of cells involved in humoral and cell-mediated immunity: consisting of $CD35^+$ dendritic cells, $CD68^+$ macrophages (antigen-presenting cells), $CD4^+$ helper T cells, $CD8^+$ killer T cells, $CD56^+$ natural killer cells and $CD138^+$ plasma B cells, where the numbers and relative proportions of each may provide additional indications of the stage and/or extent of the resulting immune responses (Figs. 6 and 7).

The overall aim of a cytokine-mediated personalized vaccination approach is supported by the available medical literature, in that the presence of tumor infiltrating lymphocytes in surgically resected tumors is generally predictive of a favorable outcome and prolonged survival in cancer patients (32); and the overall success of a given cancer immunization strategy is often associated with the presence of dendritic cells, cytotoxic T cells and plasma B cells in vaccination sites (24). The observations of the present study provide critical histological support for the concept of personalized cancer vaccination *in situ*; thus, combining the best aspects of cell-based tumor vaccines, whole-cell tumor vaccines, and cytokine-based tumor vaccines (30,31) all aimed at reshaping the host-tumor interaction in a manner that may tip the balance in favor of tumor rejection. To further advance the safety and efficacy of this personalized cancer vaccination approach to cancer immunotherapy, Epeius Biotechnologies Corp. has recently incorporated an additional fail-safe design - in the form of a suicide gene - into a second generation version of Reximmune-C-TNT, providing a clinical off switch (oral pro-drug) that would immediately ablate the entire population of GM-CSF transgene secreting tumor cells, thereby providing oncologists with a more exquisite control of the sequence and timing of the GM-CSF secretion *vis a vis* tumor cell killing.

Additional insights gained from studies of the actual pathophysiologic processes involved in 'le morte du tumour', or the death of tumors resulting from treatment with either Rexin-G or Rexin-G plus Reximmune-C, will greatly aid in the development and planning of future clinical trials using Rexin-G for tumor control, or Rexin-G plus Reximmune-C for the induction of cancer immunization. On the one hand, it might be most favorable to withhold Reximmune-C until the patient has experienced significant tumor reduction (and life extension) by the actions of Rexin-G administered as a single agent; and to bring Reximmune-C to bear largely to forestall recurrences. On the other hand, there will no doubt be cases where the observed synergism of Rexin-G and Reximmune-C will be necessitated in terms of addressing the tumor burden directly. In such cases, the histological evaluations of the desired endpoints at each point in time should be addressed with an increased sophistication of histological and radiographic evaluation criteria. We have previously shown that in the case of tumor responses to Rexin-G, wherein the primary mechanism of action is the induction of apoptosis in proliferative tumor cells and their attendant angiogenic vasculature, necrosis and cystic changes within the tumor often occur. This is due to the targeted disruption of a tumor's blood supply which starves the tumor, resulting in focal hemorrhages and additional degenerative necrosis within the tumor (Figs. 2, 4-7). In tumors of Rexin-G-treated patients, wherein apoptosis is a predominant feature, the tumors simply shrink and disappear in follow-up imaging studies (Fig. 3). However, in tumors

wherein TIL-mediated and hemorrhagic necrosis is a prominent feature, the size of the tumors may actually become larger after treatment, due to the inflammatory reactions evoked by the necrotic tumor, immune infiltrate, and cystic conversion of the tumor [Figs. 5-7; (10)]. In these cases, an increase in the size of tumor nodules on CT scan, PET scan or MRI does not necessarily indicate disease progression *per se*. Therefore, additional concomitant evaluations that closely reflect the histological quality of the treated tumors are needed to more accurately determine the extent of the necrosis and/or cystic changes induced by Rexin-G treatment. One such imaging study is the measurement of tumor density (in Hounsfield Units) in a non-contrast CT scan before and after treatment with Rexin-G or Rexin-G plus Reximmune-C. Therein, a significant reduction in tumor density from baseline measurements would be considered a favorable tumor response to treatment, even though the tumor size may increase in a more or less transient manner.

In addition to advancing an important nanotechnology/gene therapy/drug delivery platform and refining the means and methods of tumor eradication, this evaluation of the medical, histological, and cytological processes involved in 'le morte du tumour' emphasizes an aggressive yet comprehensive approach to cancer management. Clearly, the scourge of metastatic disease is no longer intractable to post-modern post-genomic medications personified by the single-agent efficacy of Rexin-G. However, the most judicial use of any medicine often calls for a strategic combinatorial approach. For example, the intense inflammatory reaction caused by massive necrosis of tumors would call for a judicious use of steroids as well as the appropriate use of a broad-spectrum antibiotic to prevent infection in the necrotic tumor nodules. Further, a program of intensive nutritional support that would expedite wound healing in necrotic tumors is imperative, particularly in patients with terminal cancer whose nutritional status are compromised. The precise sequence and timing of tumor destruction and the waveforms of GM-CSF secretion, enabled by the pro-drug sensitive construct embodied in the second generation Reximmune-C-TNT, as well as the inherent cyto-ablative potential of this new drug-sensitive construct, provide an unprecedented level of control of the tumor microenvironment that can now be deployed and examined and refined in appropriate clinical trials.

In conclusion, this study reports on several characteristic histopathologic and radiologic features of tumor cell death and histological processes associated with Rexin-G and Reximmune-C treatments, providing more definitive proofs of the precise pathotropic (disease-seeking) properties of these tumor-targeting nanoparticles bearing cytocidal and cytokine genes, respectively. Based on these findings, we advance the clinical utility and applications of pathotropic medicine in the management of metastatic cancer, we encourage the development of a novel personalized cancer vaccination protocol using a dual targeted gene transfer approach combined with a comprehensive program of supportive care, and we anticipate the refinement and elaboration of a more sophisticated set of cancer diagnostics going forward. And finally, as each new technology evolves from its inception - from a technology of the future, into a proven technology, into an enabling technology, into a disruptive technology, into a standardized technology, into a classical technology, it is important to note that all the stone tools of today will eventually become obsolete; yet the hard-fought principles of bioengineering, indeed the hard-won principles of biochemistry and biophysics, and pathotropic medicine will ultimately be incorporated into the newest of designs in the future.

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