apoptosis of the angiogenic endothelium. The fact that M21-L tumors lack αvβ3 and are not transduced by αvβ3-NP suggests that the antitumor effect is based on the antiangiogenic effects, not a direct effect on the tumor.

We next examined whether this therapy was effective against established syngeneic pulmonary and hepatic metastases of colon carcinoma. We injected murine CT-26 carcinoma cells either i.v. or intrasplenically into Balb/c mice. This experimental procedure typically results in the formation of lung or liver metastases, respectively, within four days (*19*). However, in our study, the pulmonary or hepatic metastases were established for 10 days before treatment with the NP/gene complexes to ensure that all animals contained actively growing lung or liver tumors. Control mice treated with PBS, αvβ3-NP complexed to a control vector, or nt-NP/Raf(–) showed extensive tumor burden in the lung or liver (Fig. 4, B and C) (*13*). In contrast, mice treated with αvβ3-NP/Raf(–) displayed little or no visible tumor metastasis (Fig. 4, B and C) (*13*), as demonstrated by a significant reduction in wet lung or liver weight (Fig. 4, C and D). Mice injected with αvβ3-NP/Raf(–) and a 20-fold molar excess of soluble targeting ligand had a tumor burden similar to that in control mice, demonstrating that this response is αvβ3-specific (Fig. 4, B and C). In a parallel study in which mice were killed and tumor volume was established during the course of the experiment, αvβ3-NP/Raf(–) was shown to cause regression of pulmonary metastases (Fig. 4D).

In summary, we have shown that pronounced tumor regressions can be achieved in mice by systemic delivery of an antiangiogenic gene that is targeted to the tumor vasculature. Several components of this strategy likely contribute to its pronounced antitumor activity, and these may be useful for similar treatments in humans. First, the NP used in this study has a multivalent targeting of integrin αvβ3 that selectively delivers genes to angiogenic blood vessels. A similar particle containing gadolinium and the αvβ3-targeting antibody, LM609, has been used successfully to image angiogenic blood vessels in tumor-bearing rabbits (*2*). Second, the mutant *Raf-1* gene, when delivered to these tissues, influences the signaling cascades of two prominent angiogenic growth factors, bFGF and VEGF (*13*). The robust proapoptotic activity of this gene is consistent with previous studies that have shown a role for *Raf-1* in promoting cell survival (*17*). Lastly, because NPs are less immunogenic than viral vectors, it may be feasible to deliver therapeutic genes repeatedly to angiogenic blood vessels for sustained treatment of diseases that depend on angiogenesis and vascular remodeling.

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21. Cells were exposed for 6 hours to NP electrostatically coupled to 25 μg of plasmid encoding GFP, washed with PBS, and grown in complete media. After 24 hours, cells were counterstained with 4′,6′-diamidino-2-phenylindole and fixed; GFP-expressing cells were enumerated by counting random microscopic fields. Each bar represents the mean ± SD of eight replicates.
22. FLAG-specific antibody was obtained from Zymed (South San Francisco, CA) and VE-cadherin from Santa Cruz Biotechnologies (Santa Cruz, CA). TUNEL staining was performed with the use of the Apoptag kit from Oncor (Gaithersburg, MD), and luciferase assays were performed with the use of the Luciferase assay kit from Promega (Madison, WI).
23. Weight due to tumor burden was calculated by subtracting the normal wet organ weight from each tumor-bearing organ.

24. We thank K. Spencer for assistance with immunofluorescent images and N. Alexander for assistance with vector preparation. Supported by a grant from Merck KGAa, the Lucas Foundation, the Phil Allen trust, and NIH P41 RR09784. S. Guccione is a National Cancer Institute fellow supported by NIH T32 CA09696 and CA50286. Patents are pending on αvβ3-NP and the use of ATP$^{\mu}$-Raf as antiangiogenic agents. This is TSRI manuscript number 14947-IMM.

Supporting Online Material

www.sciencemag.org/cgi/content/full/296/5577/2404/DC1
Materials and Methods
Figs. S1 to S3

24 January 2002; accepted 21 May 2002

Ongoing Modification of Mediterranean Pleistocene Sapropels Mediated by Prokaryotes

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Late Pleistocene organic-rich sediments (sapropels) from the eastern Mediterranean Sea harbor unknown, metabolically active chemoorganotrophic prokaryotes. As compared to the carbon-lean intermediate layers, sapropels exhibit elevated cell numbers, increased activities of hydrolytic exoenzymes, and increased anaerobic glucose degradation rates, suggesting that microbial carbon substrates originate from sapropel layers up to 217,000 years old. 16S ribosomal RNA gene analyses revealed that as-yet-uncultured green nonsulfur bacteria constitute up to 70% of the total microbial biomass. Crenarchaeota constitute a smaller fraction (on average, 16%). A slow but significant turnover of glucose could be detected. Apparently, sapropels are still altered by the metabolic activity of green nonsulfur bacteria and crenarchaeota.

Deep-sea sediments of the eastern Mediterranean are characterized by the cyclic occurrence of dark sediment layers, called sapropels. Sapropels differ from most other subsurface environments in that they contain high concentrations of total organic carbon (2 to 30.5% dry weight) (*1*), consisting mainly of dark brown amorphous, highly refractory kerogen (*2*). They are embedded in hemipelagic carbonate oozes that are poor in organic carbon (<0.5 weight percent) (*3*) and are likely to represent Pleistocene analogs of the widespread Mesozoic black shales (*4*). The presence of iso and anteiso fatty acids and of β-hydroxy fatty acids in the sapropels points toward a bacterial contribution to the organic matter (*5*). Because large numbers of bacteria

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and dividing cells have been detected in sapropels (*1*), the organic matter could still provide a source of carbon and energy for microbial life despite its age, and as a consequence, sapropels may still be subject to microbial alteration. To gain further insight into the fate of sapropel organic matter during diagenesis, we analyzed the composition and the physiological state of natural bacterial communities present in four sapropels (S1, S6, S7, and S8; Fig. 1, A and B), which are 8000, 172,000, 195,000, and 217,000 years old, respectively (*6*).

Acridine orange counting revealed that all four sapropels harbored large numbers of prokaryotes, which in some cases were present in microcolonies (Figs. 1C and 2A). Total cell counts (6×10^7 to 13×10^7 cells cm^{-3}) were significantly enhanced as compared to those in neighboring carbon-lean sediment layers (Fig. 2A). The frequency of dividing and divided cells in the different depths ranged between 6.7 and 13.9%. Elevated bacterial numbers and dividing cells have also been reported for older sapropels (*1*) and are a first indication of the presence of active microbial communities.

To gain more insight into the potential modification of sapropel organic matter by microorganisms, we focused on the initial steps of the anaerobic food chain, choosing hydrolytic exoenzymes as indicators of physiologically active prokaryotes. Extracellular enzymatic hydrolysis of biopolymers is the rate-limiting step for the use of organic matter in surface aquatic environments (*7*). Some exoenzymes, such as alkaline phosphatase and β-glucosidase, are subject to substrate induction and catabolite repression, so that short-term measurements of cell-specific hydrolysis rates also provide information on the actual availability of bacterial substrates (*8*, *9*). We determined the activities of the three exoenzymes alkaline phosphatase, β-glucosidase, and leucine aminopeptidase, using a method recently established for sapropels (*6*). As an additional indicator of physiological activity, we used the degradation of radiolabeled glucose to $^{14}CO_2$ (*6*). Exoenzymatic activities in sapropels are associated with intact cells (*10*).

Of the three exoenzymes, the activity of β-glucosidase was very low throughout the sediment core (Fig. 2B). The cell-specific β-glucosidase activities [0.01 to 2.3 attomoles (amol) $cell^{-1}$ $hour^{-1}$] fall well within the range observed for other marine sedi-

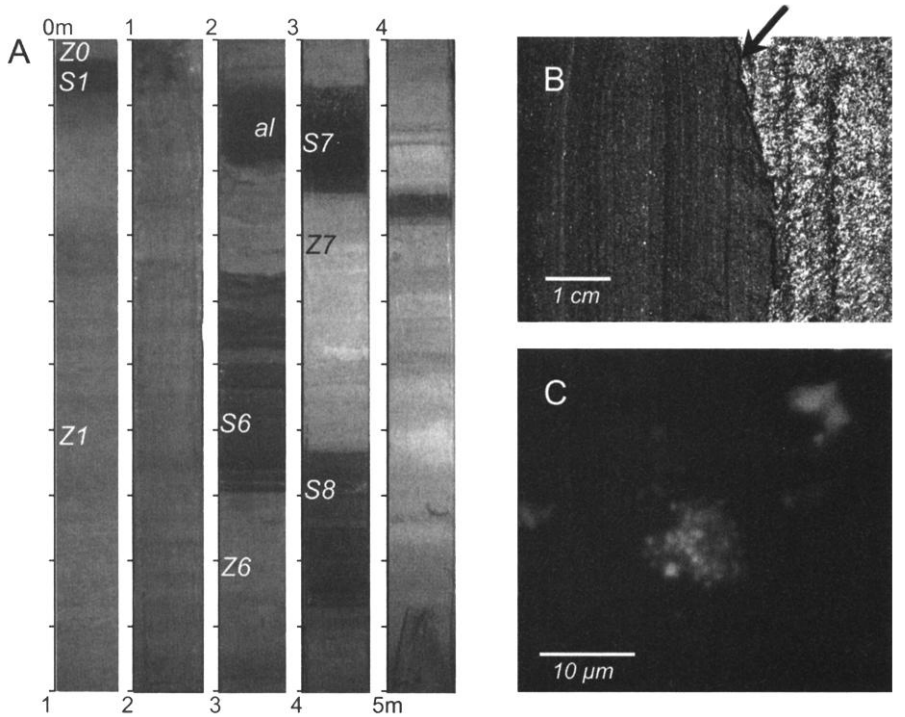

Fig. 1. (**A**) Longitudinal section of core 69-2SL. Sapropels (S1 and S6 to S8) and intermediate layers (Z0, Z1, Z6, and Z7), which were sampled for microbiological analyses, are indicated. al denotes ash layer. (**B**) Detail of the longitudinal section of sapropel S8 revealing the fine lamination after the outer layer was frozen and lifted off (to the left of the break indicated by the arrow) (*6*). The top of the sapropel is oriented toward the left. (**C**) Epifluorescence photomicrograph of a bacterial microcolony as detected in sapropel S6 after acridine orange staining.

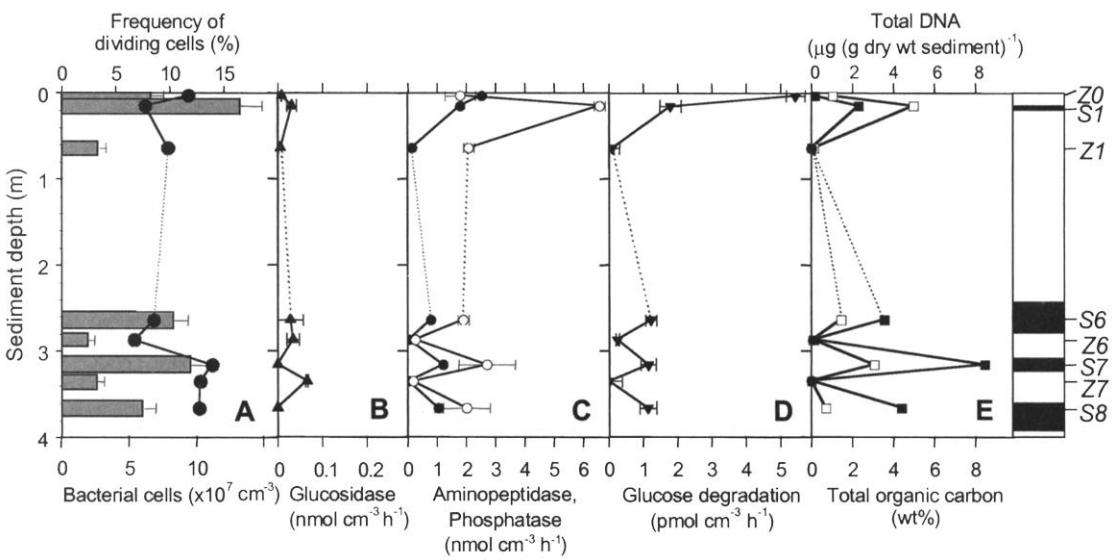

Fig. 2. (**A**) Total bacterial cell counts (horizontal gray bars) and frequency of dividing cells (●) at eight consecutive depths. Bars indicate 1 SD. (**B** to **D**) Physiological activities of microorganisms in sapropels and carbon-poor intermediate layers of core 69-2SL. Exoenzymatic activities of (B) β-glucosidase (▲), (C) aminopeptidase (●) and alkaline phosphatase (○), and (D) respiration of D-[U-^{14}C]-glucose (▼) are shown. Note the different scales of the abscissa in (B) and (C). Bars indicate 1 SD. Enzyme activities represent potential rates, because substrate analogs were used at saturating concentrations (250 μM). (**E**) Total organic carbon (■) and total genomic DNA extracted (□). The positions of sapropels and sampling depths are denoted on the right.

ments and pelagic water samples (*10*). Aminopeptidase activity and glucose degradation rates reached their highest values at the sediment surface. However, the activities of alkaline phosphatase and leucine aminopeptidase, as well as the glucose degradation rates, were consistently and significantly elevated within the sapropels as compared to intermediate layers (Fig. 2, C to D). In contrast to the sapropels, exoenzyme activities decrease rapidly with depth in other marine sediments (*11–13*), following gradients of easily degradable substances. The distinct maxima in the sapropels are therefore unexpected and cannot be explained by downward diffusion of easily degradable organic carbon. Instead, our data strongly indicate that carbon provided by the sapropel layers themselves must support bacterial metabolism in situ. This conclusion is further supported by the values of cell-specific activities of leucine aminopeptidase, which are significantly increased by a factor of 36 in sapropels S7 and S8 (10.4 to 12.0 amol cell^{-1} hour^{-1}) over those in the intermediate layers (0.2 to 0.4 amol cell^{-1} h^{-1}). These data indicate higher physiological activity of the cells in the sapropels (*10*).

Kerogens are assumed to be highly resistant to microbial attack under anoxic conditions (*14*), and adsorption can preserve intrinsically labile molecules such as amino acids and sugars against microbial degradation (*15*). The degradation of ^{14}C-glucose and the β-glucosidase activity detected suggest that glucose is one of the substrates for microbial growth in sapropels. Therefore, we determined the turnover of glucose under in situ conditions by adding radiolabeled glucose at different concentrations to anoxic sediment slurries prepared from sapropel S6 (*6*). The turnover time of glucose at in situ concentrations (21.6 μM) as calculated from this experiment was 180 days (corresponding to a first-order rate constant of degradation of k = 0.0056 day^{-1}). This value is considerably shorter than that for bulk organic matter in other marine sediments [turnover time, 537 years (*15*)], thus indicating that reactive organic carbon compounds are still present in the kerogen and still subject to microbial degradation.

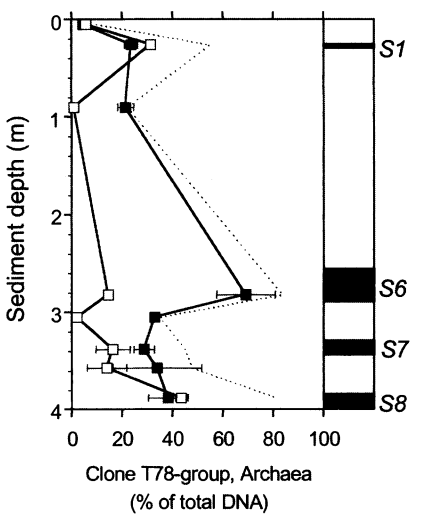

Fig. 3. Relative abundance of bacteria of the clone T78 group (■) and archaea (□) as determined by real-time PCR. Bars indicate 1 SD. The dotted line gives the sum of the abundances of green sulfur bacteria and archaea.

To identify the microorganisms potentially involved in transformations of sapropel organic matter, the dominant 16*S* ribosomal RNA (rRNA) gene sequences were analyzed. The only sediments containing old organic carbon for which bacterial communities have been characterized are Pacific deep subsurface sediments. So far, numerous barophilic sulfate-reducing bacteria and a few Proteobacteria and Crenarchaeota have been identified by molecular or cultivation-based methods (*16*, *17*).

The total amounts of DNA extracted (*6*) from the four sapropels exceeded those in the intermediate layers 5- to 250-fold (Fig. 2E). Even the 217,000-year-old Mediterranean sapropel S8 contained DNA fragments up to 30 kb long. Its high molecular weight indicates that the majority of the extracted DNA originates from extant and not subfossil prokaryotes, because hydrated DNA found in other aquatic sediments is split into short (100 to 400 base pairs long) fragments within a few thousand years (*18*).

Analyses of 16*S* rRNA genes (*6*) by polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (fig. S1), and sequencing yielded 12 partial sequences that clustered with the environmental clone T78 group of the green nonsulfur bacteria division and were distantly related to as-yet-uncultured bacteria found in deep subsurface habitats (fig. S2). Only a single sequence clustered within the δ group of the Proteobacteria, showing the greatest sequence homology (89.5%) to *Geobacter sulfurreducens* (American Type Culture Collection number 51573^{T}) (fig. S2). Amplification of archaeal 16*S* rRNA gene fragments yielded a total of 41 different phylotypes in the sapropels and intermediate layers. Most (that is, 36) of these sequences were affiliated to the group of nonthermophilic marine Crenarchaeota (fig. S3). Most notably, all but one of the 16*S* rRNA gene sequences from the sapropel layers were affiliated to this group. Archaeal DNA constituted an average of 15.9% of the total genomic DNA (Fig. 3). The abundance of archaea in the eastern Mediterranean sediment thus is higher than in other deep-sea sediments, for which values between 2.5 and 8% have been reported (*19*). By comparison, green nonsulfur bacteria constituted up to 69% of the total microbial community in sapropel S6 and significantly surpassed the abundance of archaea in five out of the eight layers investigated. On average, green sulfur bacteria and archaea together accounted for 66% of the total microbial community in sapropels and reached a maximum of 84% in sapropel S6 (Fig. 3).

With only two exceptions, none of the numerous members of the clone T78 group has ever been cultured. One isolate of the clone T78 group is *Dehalococcoides ethenogenes* strain 195, which exhibits very restricted physiological capacities and grows exclusively by oxidation of H_2, with concomitant reduction of chlorinated hydrocarbons (*20*). Recently, a thermophilic filamentous member of the clone T78 group was described, which ferments glucose or sucrose in the presence of yeast extract (*21*). Therefore, no information is available on the biogeochemical significance of nonthermophilic members of the clone T78 group (*22*). In the Mediterranean sediments, the considerable cell-specific exoenzyme activity, together with the glucose degradation, point toward a fermentative growth mode of these bacteria. So far, members of the clone T78 group have been detected in hydrothermal springs (such as the OPB clones), soil, wastewater, and subsurface environments such as paleosols (the H and TO clones, fig. S2) (*22*), but their 16*S* rRNA gene sequences usually constitute only a minor fraction (2.5%) of environmental clone libraries (*23*). The dominance of the T78 group in the Mediterranean deep-sea sediments is thus unprecedented and suggests an adaptation of these bacteria to the specific conditions in sediments containing kerogen. This view is reinforced by the fact that bacteria of the clone T78 group are much less abundant at the sediment surface than in sapropels and intermediate layers (Fig. 3).

A first insight into the mechanisms of adaptation can be obtained from a calculation of the theoretical energy demand of cells in sapropels. The lowest maintenance energy requirement of anaerobic bacteria has been determined for *Acetobacterium woodii* and is equivalent to 0.01 kJ [grams of carbon (gC) hour]$^{-1}$ (*24*). At a mean cell number of 9.3 × 10^{7} cm^{-3} in the sapropels (Fig. 2A) and a mean cell diameter of 0.7 μm, the concentration of microbial biomass amounts to 3.4 × 10^{-6} gC cm^{-3} (*25*) and its maintenance energy demand to 2.9 × 10^{-4} kJ cm^{-3} year^{-1}. Because kerogen is composed mainly of long chains of polymethylenic carbon (*14*), we used the free energy change determined for the methanogenic degradation of hexadecane of −1.95 kJ $gC_{degraded}^{-1}$ (*26*) (the corresponding value for degradation under conditions of sulfate reduction is −3.33 kJ $gC_{degraded}^{-1}$). If the prokaryotes present had a maintenance energy requirement similar to that of known laboratory strains, sapropel

organic carbon would thus be degraded at a rate of (0.88 to 1.50) $\times 10^{-4}$ gC cm^{-3} $year^{-1}$. As a consequence, even sapropels consisting entirely of organic carbon (~0.9 gC cm^{-3}) would be completely degraded within 10,000 years. Organic carbon compounds in sapropels have been preserved over much longer time intervals, although a high fraction of microbial cells in the sapropels are physiologically active and continue to use organic carbon originating from the sapropels. The above comparison thus indicates that prokaryotes in sapropels have significantly lower maintenance energy requirements than any of the pure cultures investigated to date.

Mediterranean sapropels harbor large populations of previously unknown members of the green nonsulfur bacteria and crenarchaeota. Our cumulative evidence suggests that these prokaryotes are physiologically active, are specifically adapted to the specific conditions as they prevail in sediments with large amounts of subfossil kerogen, and are capable of altering the organic matter in situ even 217,000 years after its deposition.

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27. We thank the master and the crew of the RV *Meteor* and our colleagues on board for their help during collection of the sediment cores and G. Muyzer and M. Overeijnder of the Kluwer Laboratory, TU-Delft, for help with real-time PCR. Supported by grants of the Deutsche Forschungsgemeinschaft to J.O. and H.C. (DFG Cy 1/8-1 and 1/10-1).

Supporting Online Material

www.sciencemag.org/cgi/content/full/296/5577/2407/DC1

Materials and Methods

Figs. S1 to S3

14 March 2002; accepted 17 May 2002

Correction of ADA-SCID by Stem Cell Gene Therapy Combined with Nonmyeloablative Conditioning

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Hematopoietic stem cell (HSC) gene therapy for adenosine deaminase (ADA)–deficient severe combined immunodeficiency (SCID) has shown limited clinical efficacy because of the small proportion of engrafted genetically corrected HSCs. We describe an improved protocol for gene transfer into HSCs associated with nonmyeloablative conditioning. This protocol was used in two patients for whom enzyme replacement therapy was not available, which allowed the effect of gene therapy alone to be evaluated. Sustained engraftment of engineered HSCs with differentiation into multiple lineages resulted in increased lymphocyte counts, improved immune functions (including antigen-specific responses), and lower toxic metabolites. Both patients are currently at home and clinically well, with normal growth and development. These results indicate the safety and efficacy of HSC gene therapy combined with nonmyeloablative conditioning for the treatment of SCID.

Gene therapy trials have demonstrated the safety and feasibility of engineering hematopoietic stem cells (HSCs) for treating inherited hematopoietic diseases (*1–6*). In these studies, however, the frequency of multipotent genetically modified HSCs and the levels of long-term transgene expression were variable, with limited clinical effect. This variability could be influenced by vector design, gene transfer protocols, or inadequate engraftment and expansion of genetically corrected HSCs. Recent improvements in HSC gene transfer, combined with a strong selective advantage for growth and differentiation of lymphoid cells, allowed investigators to correct the immune defect in the SCID variant due to γ-chain deficiency (SCID-X1) (*7*).

In ADA-SCID the purine metabolic defect (*8*) leads primarily to impaired lymphocyte development and function but also to nonimmunological abnormalities, which indicates that this disease is more complex than other SCIDs (*8–10*). The accumulation of toxic metabolites may offer a selective advantage to cells that produce sufficient vector-derived ADA. In previous gene therapy trials, this advantage might have been lost because of simultaneous treatment with bovine enzyme [polyethylene glycol–conjugated ADA (PEG-ADA)] replacement therapy. Recent experience with an ADA-SCID patient treated with transduced peripheral blood lymphocytes (PBL) (*11*, *12*) shows that PEG-ADA discontinuation results in preferential expansion of T cells containing the ADA gene capable of sustaining immune functions, but it did not completely correct the metabolic defect (*12*). These data suggest that, for long-term full

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