consistently expressed CD31 and reacted with BS-1 lectin.

In summary, our findings suggest that cells isolated with anti-CD34 or anti–Flk-1 can differentiate into ECs in vitro. The in vivo results suggest that circulating MB^{CD34+} or MB^{Flk1+} cells may contribute to neoangiogenesis in adult species, consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (*2*, *3*). A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (*20*) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (*21*). This issue may be successfully addressed with autologous EC transplants. The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For antineoplastic therapies, MB^{CD34+} cells could be transfected with or coupled to antitumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} cells to achieve constitutive expression of angiogenic cytokines or provisional matrix proteins or both (*22*).

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12. Single-donor human peripheral blood was obtained with a 20-gauge intravenous catheter. The first 3 ml was discarded, and the leukocyte fraction was obtained by Ficoll density gradient centrifugation. The cells were plated on plastic tissue culture for 1 hour to avoid contamination by differentiated adhesive cells.
13. MB^{CD34+}, MB^{CD34-}, and MB^{Flk1+} cells (>1 × 10^6 of each) were analyzed with anti-CD34 (Biodesign, Kennebunkport, ME) and anti–Flk-1 (Santa Cruz Biotechnologies, Santa Cruz, CA).
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18. The mean percent of DiI-labeled capillaries among total CD31-positive capillaries was determined by averaging counts made in 10 randomly selected fields (×400).
19. New Zealand White rabbits (3.8 to 4.2 kg, $n = 4$, Pine Acre Rabbitry, Norton, MA) underwent ligation of the popliteal and saphenous arteries distally, the external iliac artery proximally, and all femoral arterial branches, after which the femoral artery was excised [S. Takeshita *et al.*, *J. Clin. Invest.* **93**, 662 (1994); L. Q. Pu *et al.*, *Circulation* **88**, 208 (1993); R. Baffour *et al.*, *J. Vasc. Surg.* **16**, 181 (1992)].
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23. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a positive control. The paired primers used (sense/antisense) were as follows: for ecNOS, AAG ACA TTT TCG GGC TCA CGC TGC GCA CCC/TGG GGT AGG CAC TTT AGT AGT TCT CCT AAC [548–base pairs (bp) PCR product]; for Flk-1 (KDR), CAA CAA AGT CGG GAG AGG AG/ATG ACG ATG GAC AAG TAG CC (819-bp PCR product); for CD31, GCT GTT GGT GGA AGG AGT GC/GAA GTT GGC TGG AGG TGC TC (645-bp PCR product); for GAPDH, TGA AGG TCG GAG TCA ACG GAT TTG/CAT GTG GGC CAT GAG GTC CAC CAC (983-bp PCR product).
24. NO release was measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments, Sarasota, FL). AT^{CD34+} or AT^{CD34-} cells cultured in six-well plates were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments, Melrose Park, IL) to maintain temperature between 35° and 37°C. The sensor probe was inserted vertically into the wells, and the tip of the electrode was positioned 2 mm under the surface of the solution.

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Somatic Frameshift Mutations in the *BAX* Gene in Colon Cancers of the Microsatellite Mutator Phenotype

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Cancers of the microsatellite mutator phenotype (MMP) show exaggerated genomic instability at simple repeat sequences. More than 50 percent (21 out of 41) of human MMP^+ colon adenocarcinomas examined were found to have frameshift mutations in a tract of eight deoxyguanosines [$(G)_8$] within *BAX*, a gene that promotes apoptosis. These mutations were absent in MMP^- tumors and were significantly less frequent in $(G)_8$ repeats from other genes. Frameshift mutations were present in both *BAX* alleles in some MMP^+ colon tumor cell lines and in primary tumors. These results suggest that inactivating *BAX* mutations are selected for during the progression of colorectal MMP^+ tumors and that the wild-type *BAX* gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

The MMP pathway for colon cancer is characterized by genomic instability that leads to the accumulation of deletion and insertion mutations at simple repeat sequences (*1*–*3*). The fixation of these slippage-induced replication errors as mutations (*4*) is associated with defects in DNA mismatch repair (*5*). Colorectal MMP^+ tumors frequently contain frameshift mutations in the type II transforming growth factor–β (TGF-β) receptor gene (*6*) but are usually wild type for the *p53* tumor suppressor gene (*1*, *7*). In addition to its central role in cell growth arrest (*8*), p53 also plays a role in apoptosis in response to DNA damage (*9*). The p53 protein transactivates *BAX* (*10*), a member of the *BCL2* gene family (*11*) that promotes apoptosis (*12*).

The human *BAX* gene contains a tract of eight consecutive deoxyguanosines in the third coding exon, spanning codons 38 to 41 (ATG GGG GGG GAG) (*12*). To determine whether this sequence is a mutational target in MMP^+ tumor cells, we amplified by the polymerase chain reaction (PCR) the region containing the $(G)_8$ tract from various MMP^+ tumor cell lines. This analysis revealed band shifts suggestive of insertions and deletions of one nucleotide in some of these tumor cells (Fig. 1A). Prostate (DU145) and colon (LS180) tumor cells exhibited PCR patterns indistinguishable from those amplified from plasmids containing a *BAX* fragment with mutant $(G)_9$ and $(G)_7$ tracts (Fig. 1A, P9 and P7),

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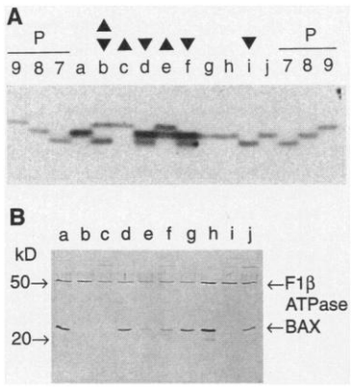

Fig. 1. (**A**) Frameshift mutations in *BAX* in MMP$^+$ tumor cell lines (*23*). PCR products of a *BAX* fragment containing the $(G)_8$ tract (*24*) are shown. SW620 MMP$^-$ colon carcinoma (lane a); LoVo, colon (lane b); DU145, prostate (lane c); LS411N, colon (lane d); SKUT1B uterus (lane e); HCT116 colon (lane f); SW48, colon (lane g); CAL51, breast, (lane h); LS180, colon (lane i); and DLD-1, colon (lane j). Two other MMP$^+$ cell lines from endometrial (AN3CA) and ovarian (SK-OV-3) cancers also showed a wild-type pattern. The triangles pointing up or down indicate the presence of insertions and deletions of one nucleotide, respectively. (**B**) Immunoblot analysis of BAX protein in MMP$^+$ tumor cell lines (*25*). Lanes are the same as in (A). Human F1β adenosine triphosphatase (ATPase) was used as an internal control for the amount and stability of cell proteins. Molecular size markers are indicated at left.

respectively. DNA derived from tumor cell lines from colon (LS411N and HCT116) and from uterus (SKUT1B) generated patterns corresponding to mixtures of the wild-type (P8) and mutant alleles. The band patterns of LoVo colon cancer cells matched those of both mutant alleles.

Immunoblot analysis (Fig. 1B) showed that LoVo, DU145, and LS180 cells, which contain no wild-type *BAX* alleles, did not express detectable amounts of BAX protein. In contrast, LS411, SKUT1B, HCT116, and tumor cells without frameshift mutations expressed various amounts of BAX. These results indicate that DU145 and LS180 cells contain homozygous (or hemizygous) frameshift insertion (DU145) and deletion (LS180) mutations in *BAX*. LS411N, SKUT1B, and HCT116 cells harbor heterozygous mutations (or are heterogeneous cell populations) for the *BAX* $(G)_8$ frameshift mutations. LoVo cells contain frameshift mutations in both *BAX* alleles, one by insertion and the other by deletion of a single nucleotide in the $(G)_8$ tract.

We next analyzed the status of *BAX* in 41 primary MMP$^+$ colon tumors. Tumor 132 contained an insertion and a deletion mutation, and tumor 197 a deletion mutation (Fig. 2A). Sequence analysis revealed that the band shifts were due to insertions $[(G)_9]$ or deletions $[(G)_7]$ of one nucleotide in the $(G)_8$ tract (Fig. 2B). *BAX* mutations were detected in 21 of 41 (51%) primary MMP$^+$ colorectal carcinomas (Fig. 3A), but not in 49 MMP$^-$ carcinomas (Fig. 3B). Thus, frameshift *BAX* mutations are specifically associated with cancer of the MMP (probability $P < 10^{-8}$, Fisher exact test). Moreover, deletion or insertion mutations in other $(G)_8$ tracts were significantly less frequent in the same MMP$^+$ tumors (*13, 14*).

We conclude that frameshift *BAX* mutations are selected for during tumorigenesis in MMP$^+$ colon tumors (*15*). The mutations in both *BAX* alleles in primary tumors, such as tumors 43, 132, and 328, indicate that they are not due to selective pressure during in vitro culture, and that in cancer of the MMP, the occurrence of biallelic mutations is not restricted to microsatellite sequences (*1*). The presence in tumor cells of inactivating mutations within both alleles of mutator or suppressor cancer genes may be diagnostic of the prior existence of the MMP (*14*). These functional double mutational hits also provide evidence that the loss of *BAX* function plays a direct role in tumorigenesis. Cells lacking BAX protein may have a diminished capacity to trigger apoptosis upon receiving a death signal. This is consistent with the phenotype of mice deficient in *BAX*, which grow normally but eventually develop lymphoid hyperplasia (*16*). Heterozygous *BAX* mutations (*15*) may also contribute to tumor progression. Inactivation of one *BAX* allele presumably reduces the amount of wild-type BAX, which may in turn facilitate escape from apoptosis by diminishing the BAX-BCL2 ratio (*17*).

In contrast to *p53* and other cancer genes that indirectly affect apoptosis, the only known function of *BAX* is to directly promote apoptosis (*11, 12, 17*). With the exception of two missense mutations detected in two human leukemia cell lines (*18*), *BAX* mutations have not been detected in human cancers. The presence of somatic *BAX* mutations in colorectal tumor cell lines and primary tumors suggests that *BAX* may be an important tumor suppressor gene in human cancer and could provide insight into the two distinct molecular genetic pathways for colon tumorigenesis (*3, 19*).

Our results may explain why colon tumors of the mutator pathway (*3*) typically do not contain *p53* mutations (*1, 7*), in contrast with those of the suppressor pathway (*19*). Once the MMP is manifested (after the occurrence of mutator mutations in, for example, DNA mismatch repair genes), mutations at the *BAX* $(G)_8$ hotspot would be more likely to occur than other frameshift or missense mutations in *p53*. In tumor cells with frameshift *BAX* mutations, transcriptional activation of *BAX* by wild-type p53 (*11*) would be irrelevant. In cancer of the MMP, the generation of thousands of DNA mismatches during every replication of each MMP$^+$ tumor cell (*20*) may trigger the p53-mediated apoptotic response to DNA damage (*21*). But this response would be futile because the chain leading to apoptosis is broken in a downstream link. Therefore, it seems reasonable to speculate that *BAX* mutations eliminate the selective pressure for *p53* mutations during colorectal tumorigenesis. Because some MMP$^+$ tumors do not have mutations in either *BAX* or *p53*, and because inactivation of these genes may be insufficient to inactivate the apoptotic pathways, escape from apoptosis may occur by other mechanisms. Although they share *BAX* mutations, some of the tumor

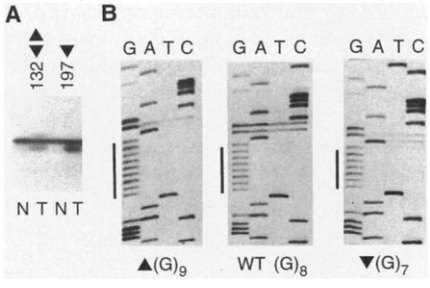

Fig. 2. PCR and sequence analysis of *BAX* mutations in MMP$^+$ primary colon tumors. (**A**) PCR products of the region spanning the $(G)_8$ tract in *BAX* from tumors (T) 132 and 197 and corresponding normal (N) tissue. (**B**) Sequences of amplified DNA fragments that showed mobility shifts (up in tumor 132 and down in tumor 197) and the normal fragment (WT) of 132 (*26*).

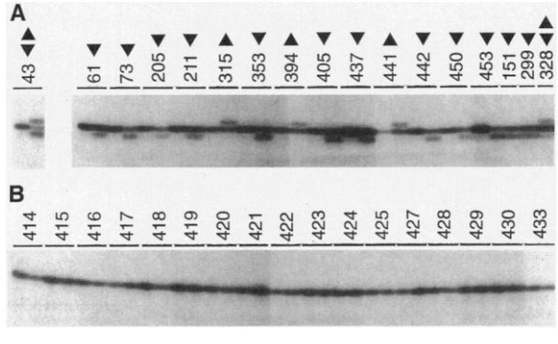

Fig. 3. Frameshift mutations in *BAX* in MMP$^+$ primary colon tumors. Representative MMP$^+$ (**A**) or MMP$^-$ (**B**) tumors (*27*). Normal tissue DNA is at the left and tumor tissue at the right in each case. Normal tissue DNA was not available for cases 151, 299, and 328. The mutations in tumor 43 were detected after microdissection from a paraffin block to enrich for tumor tissue.

cell lines used in this study appear to belong to different complementation groups for apoptosis (*22*), thus illustrating the redundant complexity of the mechanisms regulating cell death.

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13. Somatic insertion or deletion mutations in $(G)_8$ or $(C)_8$ tracts were found in the same MMP$^+$ colon tumors in the following genes: 12 of 40 in the coding region of the *hMSH6* DNA mismatch repair gene (*14*); 8 of 40 (coding) in the insulin-like growth factor II receptor gene (*IGFR-II*) [D. O. Morgan *et al.*, *Nature* **329**, 301 (1987)]; 2 of 41 in the 3′ noncoding region of the *IGFR-I* gene [A. Ullrich *et al.*, *EMBO J.* **5**, 2503 (1986)]; 2 of 41 in the promoter region of the muscle glycogen synthase (*GS*) gene [European Molecular Biology Laboratory (EMBL) and GenBank number HSGSYG1]; 2 of 40 (coding) in an uncharacterized human repair DNA protein (EMBL and GenBank number HUM51CP); 0 of 40 (coding) in a human nuclease-sensitive element DNA binding protein (*NSEP*) [R. Kolluri and A. J. Kinniburg, *Nucleic Acids Res.* **19**, 4771 (1991)]; and 0 of 41 (3′ noncoding) in the nerve growth factor receptor gene (*NGFR*) [D. Johnson *et al.*, *Cell* **47**, 545 (1986)]. The statistics (Fisher exact test) were as follows: *BAX* versus *hMSH6*, $P = 0.043$; versus *IGFR-II*, $P = 0.0032$; versus *IGFR-I*, *GS*, or *HUM51CP*, $P < 10^{-6}$; versus *NSEP* or *NGFR*, $P < 10^{-7}$.
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15. The heterozygous or homozygous nature of these mutations cannot be easily distinguished in primary tumor specimens because of the presence of variable amounts of contaminating normal tissue. The approximate amount of normal tissue could be estimated in some tumors by comparing the proportion of nonaltered versus altered sequences at several mononucleotide microsatellite loci. In this manner, we estimate that some tumors (73, 442, and 453 of Fig. 3A) harbored more mutant than normal *BAX* alleles. Microdissection experiments to select regions enriched in tumor cells revealed the homozygous nature of some mutations, which appeared heterozygous before microdissection (tumor 43, Fig. 3A). In several primary tumors (61, 211, and 437 of Fig. 3A) and in three MMP$^+$ cell lines (LS411N, HCT116, and SKUT1B), the *BAX* frameshift mutations appeared heterozygous, or present in only a fraction of the tumor cells.
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23. Tumor cell lines were obtained from the American Cell Type Collection and characterized as MMP$^+$ by PCR analysis of single-cell clones (*20, 22*). There were striking associations between *BAX* frameshift mutations and mutations in members of the *Mut S* and *Mut L* families of mutator genes, respectively. This is in line with their differences in the corresponding mutation spectra in microsatellites and cellular genes [S. Malkhosyan, A. McCarty, H. Sawai, M. Perucho, *Mutat. Res.* **316**, 249 (1996)].
24. A 94–base pair region encompassing the $(G)_8$ tract in *BAX* was amplified by PCR with primers 5′-ATC CAG GAT CGA GCA GGG CG-3′ and 5′-ACT CGC TCA GCT TCT TGG TG-3′. PCR with Vent DNA polymerase (New England Biolabs) was carried out for one cycle of 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in the presence of 0.2 mCi of [^{32}P]deoxycytidine triphosphate. PCR products were separated in a denaturing 6% polyacrylamide gel and subjected to autoradiography. P7, P8, and P9 are the PCR products amplified from plasmids containing seven, eight (wild-type), or nine G's in *BAX*, determined by nucleotide sequencing (see Fig. 2B).
25. Protein cell lysates (10 mg) were separated in a 12% SDS-polyacrylamide gel. Proteins were electroblotted onto a nylon membrane and incubated with rabbit antibodies to human BAX and F1β ATPase. Alkaline phosphatase–conjugated secondary antibodies were used for detection. S. Krajewski *et al.*, *Cancer Res.* **55**, 4471 (1995); S. Krajewski, J. M. Zapata, J. C. Reed, *Anal. Biochem.* **236**, 221 (1996).
26. The PCR products were eluted from the gels, amplified, and subcloned into pCRTM2.1 (Invitrogen). Recombinant plasmids were sequenced by the dideoxy chain termination method, with a Sequenase DNA sequencing kit (United States Biochemical). DNA was also reamplified and purified with QIAquick PCR purification kit (Qiagen) and sequenced with the ABI PRISMTM dye terminator cycle sequencing kit (Perkin-Elmer). The DNA sequences obtained by the two methods were identical.
27. Tissues were obtained from the Southern Division of the Cooperative Human Tissue Network (University of Alabama at Birmingham) as frozen specimens after surgical resections. Detailed family histories for these cases were not available. MMP$^+$ tumors were defined as those with deletion mutations in mononucleotide microsatellite loci and deletion or insertion mutations of more than one repeated unit in dinucleotide microsatellite sequences. Tumors exhibiting sporadic dinucleotide microsatellite mobility shifts of only one repeated unit without TGF-β receptor mutations were considered MMP$^-$. The properties of a subset of these tumors, were described in (*1*).

28. We thank D. Casacuberta for comments on the manuscript and G. Wahl for his gift of the CAL51 cell line. Supported by NIH grants CA63585 and CA38579. H.Y. is a postdoctoral fellow from the Japan Society for the Promotion of Science.

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Involvement of Pre- and Postsynaptic Mechanisms in Posttetanic Potentiation at *Aplysia* Synapses

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Posttetanic potentiation (PTP) is a common form of short-term synaptic plasticity that is generally thought to be entirely presynaptic. Consistent with that idea, PTP of evoked excitatory postsynaptic potentials at *Aplysia* sensory-motor neuron synapses in cell culture was reduced by presynaptic injection of a slow calcium chelator and was accompanied by an increase in the frequency but not the amplitude of spontaneous excitatory postsynaptic potentials. However, PTP was also reduced by postsynaptic injection of a rapid calcium chelator or postsynaptic hyperpolarization. Thus, PTP at these synapses is likely to involve a postsynaptic induction mechanism in addition to the known presynaptic mechanisms.

Posttetanic potentiation, an increase in synaptic strength for several minutes following high frequency stimulation of the presynaptic fibers (*1*), has been observed and extensively studied at a number of synapses including *Aplysia* sensory-motor neuron synapses (*2, 3*). *Aplysia* sensory neurons release the neurotransmitter glutamate which acts through *N*-methyl-D-aspartate–like receptors on the motor neurons, and thus these synapses may share properties with glutamatergic synapses in vertebrates (*4*). At *Aplysia* sensory-motor neuron synapses, PTP is accompanied by an increase in the frequency of spontaneous miniature excitatory postsynaptic potentials (mEPSPs) or currents (mEPSCs) with no change in their amplitude, indicating that the expression of PTP is presynaptic (*3*). In addition to PTP, repeated tetanic stimulation of the sensory neuron or pairing high-frequency stimulation of the sensory neuron with strong depolarization of the motor neuron can produce long-term potentiation (LTP) at these synapses (*5, 6*). This potentiation is reduced by postsynaptic hyperpolarization during the tetanic stimulation or infusion of the Ca^{2+} chelator BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] into the postsynaptic neuron, indicating that the induction of LTP involves postsynaptic events. Therefore, we