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J Clin Invest. 1997;100(9):2182-2188. https://doi.org/10.1172/JCI119754.

Research Article

Cells proliferating from human atherosclerotic lesions are resistant to the antiproliferative effect of TGF-beta1, a key factor in wound repair. DNA from human atherosclerotic and restenotic lesions was used to test the hypothesis that microsatellite instability leads to specific loss of the Type II receptor for TGF-beta1 (TbetaR-II), causing acquired resistance to TGF-beta1. High fidelity PCR and restriction analysis was adapted to analyze deletions in an A10 microsatellite within TbetaR-II. DNA from lesions, and cells grown from lesions, showed acquired 1 and 2 bp deletions in TbetaR-II, while microsatellites in the hMSH3 and hMSH6 genes, and hypermutable regions of p53 were unaffected. Sequencing confirmed that these deletions occurred principally in the replication error-prone A10 microsatellite region, though nonmicrosatellite mutations were observed. The mutations could be identified within specific patches of the lesion, while the surrounding tissue, or unaffected arteries, exhibited the wild-type genotype. This microsatellite deletion causes frameshift loss of receptor function, and thus, resistance to the antiproliferative and apoptotic effects of TGF-beta1. We propose that microsatellite instability in TbetaR-II disables growth inhibitory pathways, allowing monoclonal selection of a disease-prone cell type within some vascular lesions.

Genomic Instability in the Type II TGF-β1 Receptor Gene in Atherosclerotic and Restenotic Vascular Cells

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Abstract

Cells proliferating from human atherosclerotic lesions are resistant to the antiproliferative effect of TGF-β1, a key factor in wound repair. DNA from human atherosclerotic and restenotic lesions was used to test the hypothesis that microsatellite instability leads to specific loss of the Type II receptor for TGF-β1 (TβR-II), causing acquired resistance to TGF-β1. High fidelity PCR and restriction analysis was adapted to analyze deletions in an A_{10} microsatellite within TβR-II. DNA from lesions, and cells grown from lesions, showed acquired 1 and 2 bp deletions in TβR-II, while microsatellites in the hMSH3 and hMSH6 genes, and hypermutable regions of p53 were unaffected. Sequencing confirmed that these deletions occurred principally in the replication error-prone A_{10} microsatellite region, though nonmicrosatellite mutations were observed. The mutations could be identified within specific patches of the lesion, while the surrounding tissue, or unaffected arteries, exhibited the wild-type genotype. This microsatellite deletion causes frameshift loss of receptor function, and thus, resistance to the antiproliferative and apoptotic effects of TGF-β1. We propose that microsatellite instability in TβR-II disables growth inhibitory pathways, allowing monoclonal selection of a disease-prone cell type within some vascular lesions. (*J. Clin. Invest.* 1997. 100:2182–2188.) Key words: microsatellite instability • cardiovascular disease • TGF-β • receptors • DNA mutations

Introduction

Occlusive vascular disease is the major risk for myocardial infarction and stroke, leading to more than 500,000 deaths per year. Vascular lesions are thought to result from an excessive wound healing response to chronic vascular injuries such as hypertension, hyperlipoproteinemia, and smoking (1). However, the factors that distinguish normal from pathological wound repair remain poorly defined. Serial angiographic studies indicate that most patients undergoing balloon angioplasty, a defined vascular injury, show a restenotic narrowing of the artery because of fibroproliferative intimal hyperplasia. Surprisingly, many patients spontaneously resolve the intimal lesion to achieve a net luminal gain, while the remaining 35–40% progress to clinical restenosis (2). The fibroproliferative response to acute vascular injury is characterized by migration of smooth muscle–like cells (SMC)[1] or myofibroblasts into the arterial intima and subsequent proliferation and extracellular matrix synthesis. Under normal conditions, however, the intimal lesion regresses through apoptosis of the intimal cells (3, 4).

Thus, vascular lesions could result from either excessive fibroproliferative signals, or as suggested by Rembold (5) and others, from reduced apoptotic signals for lesion resolution. TGF-β1 is known to control both the positive and negative regulatory systems involved in the wound healing process: cell proliferation/inhibition, migration, extracellular matrix synthesis, and apoptosis (6), and TGF-β1 is highly expressed in primary and restenotic vascular lesions (7). While TGF-β1 is often regarded as a purely profibrotic factor, considerable evidence indicates that TGF-β1 can exert diverse, and potentially protective effects within the vascular wall. Based on its ability to inhibit the proliferation and migration of vascular cells, TGF-β1 has been characterized as central in a protective cytokine theory of vessel wall disease (8, 9). This theory is supported by significant animal and human data in which alterations in active TGF-β1 levels were associated with the progression of vascular disease in a manner consistent with a protective effect of TGF-β1 (10, 11).

Previous studies have used cells grown from primary and restenotic vascular lesions to study their response to injury-induced signals, such as TGF-β1, as a model of the atherosclerotic/restenotic process (6, 12). Although TGF-β1 is a potent growth inhibitor for normal human SMC (13), cells derived from human atherosclerotic and restenotic lesions are resistant to the antiproliferative effect of TGF-β1 (6). Resistance to TGF-β1 in the lesion-derived cells is associated with decreased membrane expression of the Type II receptor, detected by radioligand cross-linking, and with decreased levels of the Type II, but not the Type I, mRNA by reverse transcriptase (RT)-PCR (6). Furthermore, transfection of Type II receptor cDNA restores the antiproliferative response, suggesting that the intracellular signaling systems remain largely intact. Related studies in aged rats, which develop excessive injury-induced intimal hyperplasia, observed that the SMC of aged animals have acquired resistance to the antiproliferative effect of TGF-β1, and have lost specific TGF-β1 receptors (14).

TGF-β1 induces diverse cellular changes through hetero-

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Received for publication 1 May 1997 and accepted in revised form 18 September 1997.

J. Clin. Invest.

0021-9738/97/11/2182/07 $2.00
Volume 100, Number 9, November 1997, 2182–2188
http://www.jci.org

1. *Abbreviations used in this paper:* SLP, strand length polymorphism; SMC, smooth muscle cells; TβR-II, TGF-β receptor Type II; WT, wild type.

meric complexes of Type II and Type I receptors, such that the loss of either receptor dramatically alters the cellular response (15). Mutations in two microsatellite regions of the Type II receptor gene can result from a replication error–prone (RER+) phenotype in hereditary nonpolyposis colon carcinoma (HNPCC) (16, 17). The A_{10} microsatellite, which is within the coding region of the extracellular domain of the Type II receptor, is prone to 1 bp deletions that introduce a stop codon ~ 100 bp downstream of the mutation, leading to a reduction in gene expression, and resistance to TGF-β1's antiproliferative effect (16). Because TGF-β1 can be a potent, autocrine growth inhibitor for a variety of cells, including SMC, an acquired mutation in the Type II receptor would confer a relative growth advantage on the cells bearing the mutation, allowing monoclonal, or oligoclonal, selection in a given tissue. The monoclonal nature of many cancers is well-documented (18), and previous data analyzing mosaicism (19, 20) and chromosomal abnormalities (21) in humans, have demonstrated monoclonal expansions in atherosclerotic lesions (for review see reference 22).

These findings led us to hypothesize that the abnormal receptor profile and aberrant response to TGF-β1 observed in cells derived from atherosclerotic lesions may result from microsatellite instability in the Type II receptor for TGF-β1 (TβR-II). This hypothesis was tested by examining microsatellite instability in atherosclerotic lesions, restenotic lesions, cells cultured from both lesion types, as well as normal human arteries.

Methods

Patients. Vascular specimens were acquired as waste surgical material, under IRB-approved protocols, from patients undergoing percutaneous directional coronary atherectomy (DCA), surgical endarterectomy, or coronary artery bypass grafts (CABG) at The New York Hospital/Cornell Medical Center. Portions of the vascular lesions were explanted for cell culture and the remaining specimens were stored at −70°C until DNA was purified.

Cells. Cells were cultured by explant from human carotid and femoral/iliac endarterectomies, as well as from coronary artery atherectomy specimens as described previously (6, 12). Cells were cultured in Medium 199 with 10% FBS and 50 μg/ml gentamicin sulfate (GIBCO BRL, Gaithersburg, MD). DNA was typically purified within two to four passages (4–6 wk) after isolation of the tissue. The response of these cells to TGF-β1, with respect to α-actin expression, collagen production, plasminogen activator inhibitor-1 (PAI-1) activity, and cell proliferation, as well as to TGF-β1 receptor status by radioligand cross-linking and RT-PCR, has been reported previously (6).

PCR. DNA was purified from tissues and cells by proteinase K/SDS digestion, phenol/chloroform extraction, and RNAse digestion. The human Type II receptor sequence was reported by Lin et al., Genbank accession no. M85079 (23). PCR primers spanning the A_{10} microsatellite region (709–718) were synthesized to anneal with positions 631–650 and 745–768 yielding a 138-nt product. Exact primer sequences are available from the authors. Standards containing the known wild-type or mutant sequence were subcloned in PCR-II (Invitrogen Corp., San Diego, CA) by A/T cloning, expanded in DH5α plus bacteria, and confirmed by dideoxy terminator sequencing.

SLP. For detecting strand-length polymorphisms (SLP) in the Type II receptor, 200 ng of DNA was amplified by PCR using 0.5 μg of each primer, 2.5 U of *Pfu* polymerase (Stratagene, Inc., La Jolla, CA), 50 μM dNTPs, 1 μCi of [^{32}P]CTP or [^{35}S]ATP, and 2 mM $MgSO_4$ for 30 cycles at 60°C annealing/72°C extension. Amplifications were visualized with ethidium bromide on an agarose gel, and inefficient amplifications were excluded from further consideration. The radiolabeled product was then cut with 4 U of Alu1 (New England Biolabs, Inc., Beverly, MA) for 1 h at 37°C, yielding blunt ends at positions 651 and 751 (101-bp fragment). The products were separated on a 6% acrylamide/8 M urea sequencing gel (2,000 V for 4–6 h). The gel was vacuum dried and exposed to Kodak XAR film and a storage phosphor screen (Storm; Molecular Dynamics, Sunnyvale, CA).

The mutation rate was quantified by measuring the radioactivity in the mutant band (100 bp) as a percentage of the WT (101 bp) plus mutant, thus compensating for minor loading variations. In SLP assay of genomic standards, human umbilical cord DNA (WT, A_{10}) or HCT116 colon carcinoma (American Type Culture Collection, Rockville, MD) DNA (MUT, A_9), typically 10% of the counts are found in the MUT band when the template was WT, and as much as 15% of the counts appeared WT when the template was MUT. This 10–15% error rate is due to (*a*) background counts in the lane, and (*b*) deletions or additions created by PCR. This background error rate was subtracted from all samples, and thus the reported mutation rate is relative to the standards for each experiment. Control reactions containing no patient DNA (H_2O) were consistently negative.

Control studies using genomic DNA mixed at various WT/MUT ratios indicated that the assay system was linear over the range of 0–100% ($r = 0.986$), but tended to underestimate the true mutation rate by up to 10% at low mutation levels. Duplicate amplifications indicated that the assay had a 12% coefficient of variance across the full range. Dilutions of input DNA (20–200 ng) were examined and indicated that the apparent mutation rate did not deviate from the predicted value even at 10-fold lower levels of input DNA.

DNA sequencing. The primers used for SLP were modified with M13 (−21, forward and reverse) primer sequences at their 5′ ends, and 100 ng of genomic DNA was amplified in a two stage profile (8 cycles at 60°C, 32 cycles at 70°C). The product was checked with agarose gel electrophoresis and then sequenced by fluorescent dye-primer dideoxynucleotide terminator chemistry using AmpliTaq FS (Perkin-Elmer, Foster City, CA), and the labeled products were analyzed on an ABI 373A automated sequencer. Alternatively, in the case of principally wild-type or mutant samples, the PCR products were tailed with *Taq* polymerase to facilitate A/T subcloning into PCR-II. The purified plasmids were then sequenced by conventional fluorescent dye-terminator chemistry.

Results

Diagnosis of TGF-β1 receptor mutations by SLP. The slippage-prone nature of microsatellite regions presents technical challenges in their diagnosis because: (*a*) some polymerases used in PCR slip on repeated regions, and in particular, poly A regions; (*b*) the terminal nicking (*Pfu*) or tailing (*Taq*) activity of PCR polymerases alters strand length in an unpredictable manner; and (*c*) primer length impurities create variations in product length. These problems were dramatically reduced by: (*a*) the use of a high fidelity polymerase (*Pfu*), and (*b*) double blunt restriction (*Alu1*) to eliminate variations from primer length and polymerase nicking. Human umbilical cord DNA was used as a wild-type control (WT, A_{10}) and the HCT116 colon carcinoma cell line was used as a known mutant (MUT, A_9).

Microsatellite analysis of a representative set of patients in which both the atherosclerotic tissue (*T*) and cells (*C*) were available demonstrates several important effects (Fig. 1). First, the mutation in the Type II receptor is present at a significant level in the lesions of a subset of endarterectomy patients as demonstrated by patient E75 which shows a 34% mutation rate in the tissue (Fig. 1 *A*). Second, in a much larger fraction of patients, the mutation rate is higher in the cells grown from the lesion than in the original tissue, suggesting either that

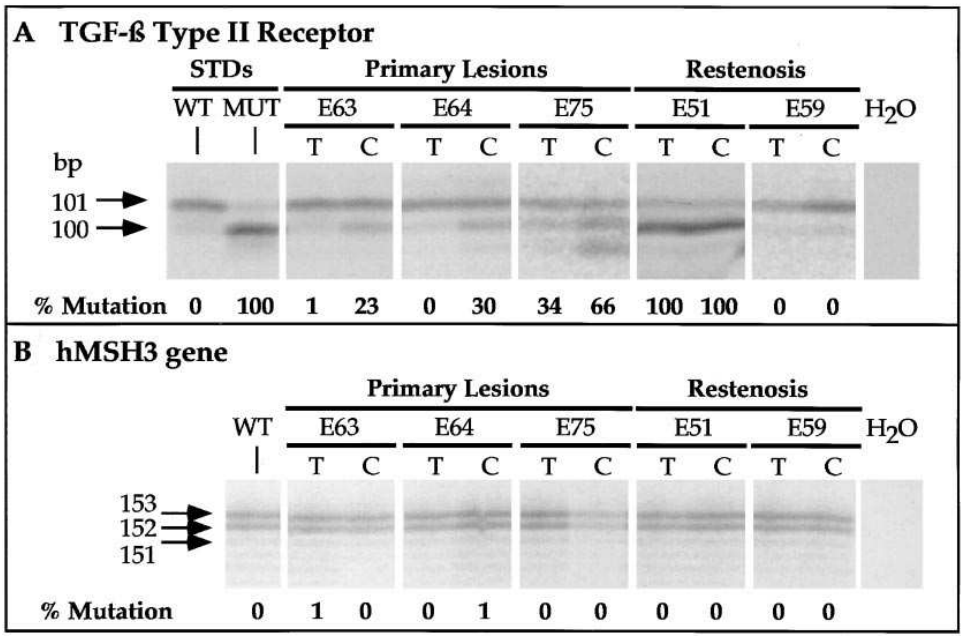

Figure 1. Analysis of microsatellite instability in the Type II TGF-β1 receptor in atherosclerotic tissues and cultured cells by SLP. DNA was prepared from atherosclerotic tissue (*T*) or cells (*C*) cultured from the plaque of five patients undergoing carotid artery endarterectomy (*E63*, *E64*, etc.). (*A*) Standards (*STDs*) of genomic DNA exhibiting the mutant (*MUT*, A_9, HCT116) or the wild-type (*WT*, A_{10}, human umbilical cord) sequence were used to calibrate migration and sensitivity. A 138-bp fragment containing the microsatellite region of the Type II receptor was amplified with *Pfu* polymerase and [^{32}P]CTP. The product was digested with Alu1, yielding an internal, blunt-end fragment of 101 bp (*WT*), containing the microsatellite, and two small terminal fragments (not shown). The PCR product was denatured by boiling with formamide and separated on a sequencing gel (6% polyacrylamide, 8 M urea). Mutation rate was quantitated by autoradiography on a storage phosphor screen. Reactions in the absence of patient DNA were negative (H_2O). (*B*) The microsatellite region in the hMSH3 gene, containing an A_8 region, was analyzed essentially as described above, without Alu1 restriction, using primer pairs described previously (24). Mutation rates are expressed relative to the genomic standards for each experiment.

these replication errors are acquired in vitro, or that a small, undetectable subset of mutant cells present in the tissue expand in cell culture. In particular, patient E75 is striking in that the tissue shows a 34% mutation rate, while cells derived from the lesion show a 66% error rate in the first passage, nearly half of which is due to a second deletion mutation to A_8.

Two patients in which the initial endarterectomy was successful, but which slowly reoccluded because of fibroproliferative regrowth (restenosis), were also examined. In both cases, cells cultured from the lesions were available for analysis (E51, E59). Patient E51 exhibited an essentially pure mutant genotype in the tissue, and the mutation was retained by the cultured cells. In contrast, patient E59 exhibited a wild-type genotype that was retained by the cultured cells. PCR products from E51T and E59T were subcloned and sequenced to confirm that the deletion was within the A_{10} region (not shown). Thus, while a mutation at this locus can occur at very high rates in restenotic tissues, it is not necessary for restenosis to occur.

In order to control for the possibility that random slippage of the *Pfu* polymerase created the apparent mutations, and to address the extent and specificity of the microsatellite errors, a subset of the samples was also analyzed for the presence of microsatellite, and nonmicrosatellite mutations in other genes. The human mismatch repair enzymes, hMSH3 and hMSH6, were selected because they contain similar microsatellite regions, and because they are thought to play a role in the etiology of the RER+ phenotype (24). SLP analysis of the hMSH3 gene, containing an A_8 microsatellite, was conducted without double-blunt restriction and thus two major bands are visible because of polymerase nicking (Fig. 1 *B*). Analysis of SLP pattern revealed a low and invariant level of deletion mutants that were indistinguishable from a pure, plasmid control (WT). Similar results were obtained with the hMSH6 microsatellite (C_8) (not shown). Additionally, other patient samples were analyzed for the status of the p53 gene using an RNAse mismatch cleavage assay (Ambion, Inc., Austin, TX) spanning exons 5–9 of the p53 gene. While p53 mutations can cause TGF-β1 resistance (25), no evidence of p53 mutations could be detected in samples from five patients, a finding corroborated by other published reports in lesions derived from 32 patients (26).

Vascular distribution. The previous data documents the distribution of the receptor mutations in carotid artery atherosclerotic lesions. Atherosclerotic lesions from coronary arteries, retrieved by DCA, were also analyzed. As shown in Fig. 2, primary (de novo) coronary lesions exhibited a range of mutation rates from completely wild-type (A359) to largely mutant (A221). In a preliminary analysis of restenotic specimens (patients who reoccluded after a previous angioplasty), an equally diverse mutation rate was observed (2–100%). An accurate assessment of the relationship between the mutation rate and disease progression after angioplasty will need to evaluate both the clinical follow up on de novo lesions, which include both patients with and without subsequent restenosis, and will need to evaluate the integrity of entire Type II TGF-β receptor gene.

By contrast, an analysis of grossly normal internal mammary arteries acquired during CABG indicated that microsatellite instability could not be detected, despite the roughly similar age and general health of these patients (Fig. 2 *B*). This suggests that the mutations accumulate relatively specifically in atherosclerotic tissues, a finding supported by a subsequent analysis of microdissected regions of atherosclerotic arteries (Fig. 3).

Sequence analysis. In order to confirm the identity of mu-

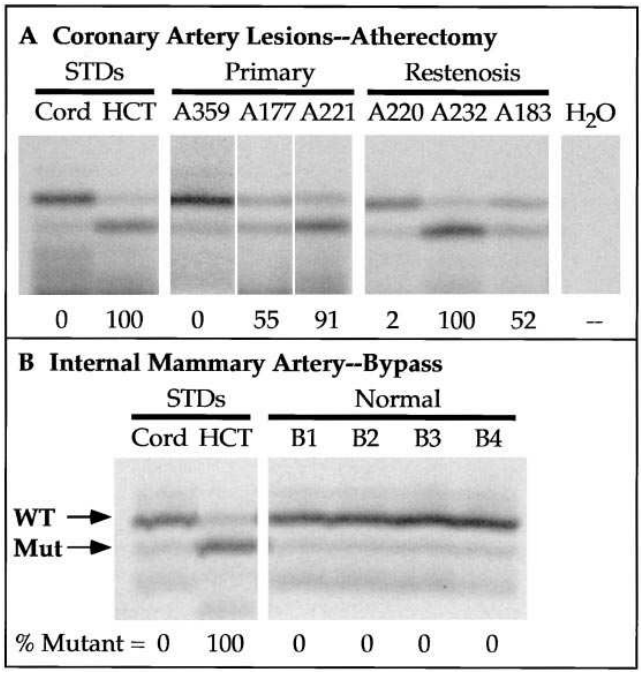

Figure 2. Analysis of microsatellite mutations in primary and restenotic coronary artery lesions and normal internal mammary artery. (*A*) Tissue specimens were collected from human coronary artery lesions by directional atherectomy, and DNA was analyzed as described in Methods. Mutation rates were quantitated from storage phosphor screens and expressed relative to genomic standard of human umbilical cord (*Cord*, WT) or HCT116 (*HCT*, Mut). PCR reactions without DNA (H_2O) were negative. (*B*) Grossly normal internal mammary arteries were collected from coronary artery bypass operations and analyzed by the same methods.

tations diagnosed by PCR and SLP analysis as deletions in the A_{10} region, PCR products were sequenced by dye-primer/dideoxynucleotide sequencing. PCR products were directly sequenced with a mutant *Taq* enzyme that efficiently incorporates dideoxynucleotides and a modified polymerase cocktail containing a thermostable pyrophosphatase. This method was used to sequence the hMSH2 microsatellite (A_{25}) with high fidelity (27). Using this modified sequencing technique, it was observed that in patients exhibiting mutations in the SLP assay, the Type II receptor microsatellite region had significant evidence of deletion mutants in the A_{10} region. Sequencing of patient specimens that were also analyzed by SLP indicates a strong positive correlation ($r = 0.93$, $n = 7$) in the apparent mutation rate determined by the two different methods, thus further obviating the possibility that the mutations are caused by random slippage of polymerase, which would create random mutation rates in any two separate amplifications of the same specimen.

Additionally, the PCR products from patients exhibiting strongly wild-type or mutant genotypes by SLP were also subcloned into PCR-II and sequenced by fluorescent dye–terminator chemistry. Because the PCR product can insert into the plasmid in either orientation, the sequences are randomly derived from the sense or antisense strand. The results, shown in Fig. 4, indicate that the observed deletions occurred within the A_{10} microsatellite region. Patient A220 which showed only a 2% mutation rate by SLP (Fig. 2), exhibited an intact A_{10} microsatellite by sequencing (Fig. 4, *top*). Conversely, patient A232, which showed an essentially pure mutant genotype by SLP (Fig. 2), exhibited the A_9 genotype by sequencing. Identical results were obtained by sequencing of the E51T (Mut) and E59T (WT) PCR products from the endarterectomy patients shown in Fig. 1.

Point mutations. In HNPCC cell lines, both microsatellite errors and missense mutations have been observed (28). In the course of examining the microsatellite sequences by SLP and fluorescent sequencing, it was observed that, in some patients, flanking sequences exhibited point mutations or restriction polymorphisms. These mutations could be confirmed by the availability of adjacent normal tissue, or in the case of cells, by the availability of the original tissue, which exhibited the wild-type sequence. In one particular case, patient E26, the con-

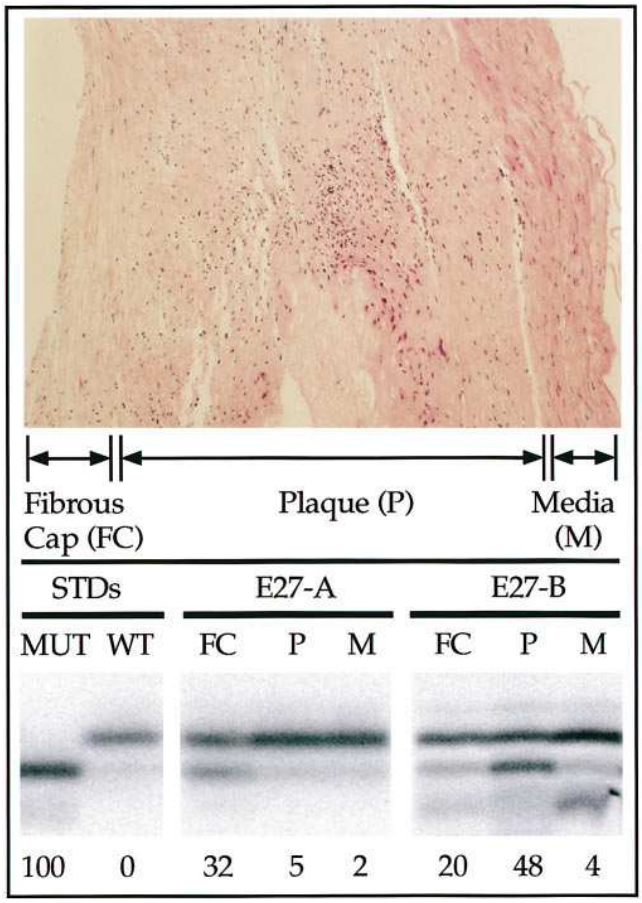

Figure 3. Intralesion distribution of microsatellite mutations. A single, primary atherosclerotic lesion, recovered by carotid endarterectomy, was dissected into regions containing the fibrous cap (*FC*) of the lesion at the luminal surface, the calcified, cholesterol-rich plaque (*P*) core, and the base of the lesion containing principally the underlying media (*M*). In the upper panel, an adjacent hematoxylin/eosin-stained section shows the approximate demarcations of the anatomical regions used. In the lower panel, two adjacent 0.5 cm sections of the lesion (*E27-A* and *E27-B*) were analyzed by SLP as described. DNA standards were isolated from HCT116 (*MUT*), a colon carcinoma cell line, or human umbilical cord (*WT*). Mutation rates (below each lane) were quantitated from exposure to storage phosphor screens, and expressed relative to the genomic standards.

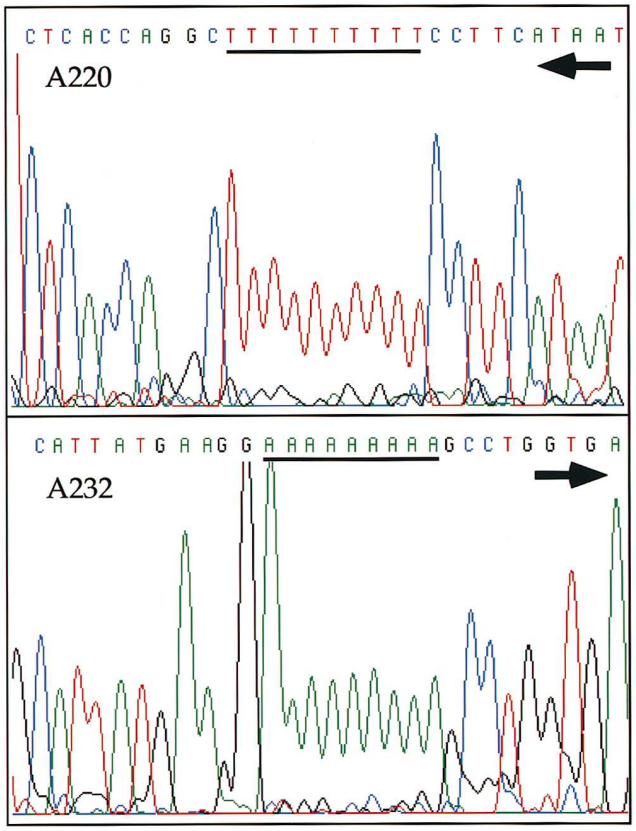

Figure 4. Sequence analysis of the microsatellite region of the Type II TGF-β1 receptor. DNA from two patient tissue specimens shown in Fig. 2 was analyzed by subcloning the PCR product into PCR-II, followed by fluorescent dideoxy sequencing using M13 primer sites contained in the plasmid. Dye-labeled products were analyzed on an ABI 373A automated sequencer and show a 30-bp region spanning the A_{10} region of the Type II receptor. The PCR products insert into the plasmid randomly in either orientation (*bold arrows*) such that patient A220 DNA shows the wild-type microsatellite (A_{10}) in the reverse and complementary sequence (T_{10}), and patient A232 shows the forward cDNA sequence except for the presence of the microsatellite mutation (A_9).

tralateral carotid plaque was available for analysis (E27, Fig. 4). It was determined that both the E26 tissue and cells grown from the plaque exhibited a point mutation (A→G) at position 790 leading to a Glu to Gly transition in the amino acid sequence. This mutation was not present in the contralateral artery, suggesting it was a true mutation and not a polymorphism. Other patients were observed, such as E75, in which regions of the plaque core or the fibrous cap exhibited point mutations not present in the remainder of the lesion. In this case, the mutation rate could be quantified because it inactivated the 5′ Alu1 restriction site at position 651, leading to an altered restriction pattern. Cells derived from patient E88 showed a similar Alu1 restriction mutation that was not detectable in the tissue. Likewise, cells derived from patient E47 showed an essentially pure monoclonal mutation at the 5′ Alu1 site that was determined, by sequencing, to result from a 2-bp substitution inactivating the site. Thus, combined with the microsatellite errors, these point mutations strongly suggest that lesions, and cells deriving from lesions, contain monoclonal expansions of cells with acquired mutations.

Intra-plaque patch analysis. The relationship of the microsatellite mutation to the pathology of atherosclerosis was further evaluated in a subset of patients in which the large carotid lesions were dissected into three specific regions: (*a*) the base of the lesion, which in the patient shown in Fig. 3 (E27) appeared to contain principally a portion of the normal media (*M*); (*b*) the cholesterol-rich, calcified core of the plaque (*P*); and (*c*) the fibrous cap (*FC*), containing a predominantly smooth muscle–like layer with the overlying endothelium (Fig. 3, *top*). Microdissection was conducted on two adjacent 0.5-cm sections through the specimen (*E27-A* and *E27-B*) and the DNA was analyzed by SLP. Specimen E27-A exhibits the wild-type genotype in both the media and plaque, while the overlying fibrous cap exhibits a high (32%) mutation rate. Analysis of adjacent regions of the lesion (*E27-B*) indicated that the patch of mutant cells persisted in the fibrous cap (20%) and could be followed into the plaque core where a mutation rate of 48% was observed, while the media remained normal. The contralateral artery of this patient was wild type at the microsatellite region, but exhibited a point mutation in a flanking region, as discussed. Thus, in this patient, two findings become evident: (*a*) these mutations are clearly acquired in nature, and (*b*) the mutations can be tracked both laterally and radially as patches containing a high proportion of mutant cells.

Of nine lesions, from eight patients, three lesions showed a similar pattern of patches containing 30–50% mutant alleles, with the adjacent tissue being unaffected. Five patients showed 10–20% mutant alleles with relatively minor differences between regions, and only one patient showed no evidence of mutations at these microsatellite loci. An expanded analysis of other mutations in this gene, and other growth/apoptotic modulators, will be required to evaluate fully the role of genomic instability in disease progression.

Discussion

The presence of acquired mutations in the Type II receptor within cells composing the atherosclerotic lesion might provide new theoretical and experimental avenues for understanding cardiovascular disease progression. The data suggest that monoclonal expansions of mutant cells exist within some atherosclerotic lesions, a finding that has been observed for more than 20 yr without a satisfying explanation for the mechanism of the monoclonal expansion (19, 22). The acquired resistance to TGF-β1 offers one potential mechanism by which monoclonal expansion could occur, and raises interesting questions about the nature of the expansion. Our results suggest that the mutant cell is smooth muscle/myofibroblast in nature, by virtue of its ability to expand in cell culture and express the contractile actin isoform (6). This fact, however, does not exclude the possibility that cells of the macrophage and lymphocyte lineages could also acquire mutations, nor does it address whether the mutant cells derive from cells in the media, adventitia, or circulation.

Further evidence for the occurrence of microsatellite instability and loss of heterozygosity in atherosclerotic lesions has been reported recently (29). In that case, 20% of lesions showed mutations in at least one of seven random microsatellite regions. Our data demonstrates that instability can occur at a microsatellite region that offers a selective advantage to the cell: the Type II receptor for TGF-β1. This type of mutation

provides the cells limited immunity from the antiproliferative (6) and apoptotic effects of TGF-β1 (30), and possibly from other regulatory factors that act partially through TGF-β1, such as heparin (31–34).

This analysis probably underestimates the true frequency of TGF-β receptor mutations for several reasons. First, another unstable microsatellite region has been documented in colon carcinoma: two copies of a dinucleotide repeat (GTG-TGT) that, when expanded, leads to an altered COOH-terminal amino acid sequence for the receptor (16). Likewise, mutations in the promoter for the Type II receptor can lead to loss of receptor expression, as demonstrated in squamous cell carcinoma (35). In other cell types, Type I receptor mutations have been reported that lead to TGF-β1 resistance (36). Non-microsatellite, random mutations in other genes are known to accumulate during the normal aging process (37), occurring frequently when methylcytosine is oxidatively deaminated to thymidine (38). Potentially, there are other components of the TGF-β1 signaling system that, if mutated, would confer partial or complete resistance to TGF-β1's effects. By extension, it seems likely that other antiproliferative or apoptotic genes could acquire mutations, thereby conferring resistance and monoclonal expansion. It is also important to consider the possibility that nonmutagenic events, such as viral infection (39) or overexpression of antagonists (40), could confer resistance to TGF-β's antiproliferative effects.

The possibility that inhibitor resistance results from acquired mutations is consistent with the known risk factors in the pathogenesis of vascular disease. It is well-established that increasing age is the single strongest risk for cardiovascular disease (41). Although conventional risk factors for atherosclerosis are poor predictors of restenosis after angioplasty, increasing age is a positive predictor of restenosis, such that restenosis is 50% more likely above age 65 than below (42). The effect of age on disease progression is poorly understood, but is often attributed to the cumulative effect of repeated vascular insult. However, the precise mechanism by which repeated injuries accumulate to predispose an artery to disease is not known. Based on our data, we propose that the cumulative effect of arterial injury is reflected in the genomic fidelity of the cells that respond to the injury. A common feature of cardiovascular risk factors such as smoking, hyperlipidemia, and hypertension is that they would be expected to increase cell turnover, and thus increase the probability of replication errors in DNA in the affected vascular region. The plaque environment, as a chronic inflammatory site, may elaborate a variety of endogenous mutagens such as free radicals. Furthermore, exogenous risk factors, such as smoking, might directly contribute to DNA modifications at hypermutable sites via free radicals and reactive aromatic hydrocarbons (43). In light of the increasing role of genomic instability in various malignancies, these data raise important questions about the role of genomic instability in other nonmalignant disorders characterized by focal growth abnormalities.

Acknowledgments

The authors are grateful for the support of the National Institutes of Health/National Institutes on Aging RO1 (AG12712) and the National Institutes of Health/National Heart, Lung, and Blood Institute SCOR in Atherosclerosis (HL56987), without which this work would not have been possible.

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