Ischemia-Induced K-ras Mutations in Human Colorectal Cancer Cells: Role of Microenvironmental Regulation of MSH2 Expression

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Abstract

Mutation of the K-ras gene is one of the most common genetic alterations in solid tumors, including colorectal cancer. The relatively late emergence of K-ras mutations in colorectal cancer is particularly striking in the class of mismatch repair–deficient tumors associated with early-onset microsatellite instability. We, therefore, tested the hypothesis that the microsatellite instability phenotype itself does not efficiently trigger K-ras mutations in colorectal cancer cells, but rather that tumor-associated microenvironmental conditions (e.g., hypoxia and hypoglycemia) contribute to this event by modulating genetic instability. We examined K-ras \textit{G13D} mutation using PCR-RFLP analysis in two different microsatellite instability colorectal cancer cell lines (HCT116 and DLD-1) and their variants in which the mutant (but not the wild-type) K-ras allele has been genetically disrupted (Hkh-2 and Dks-8). We found K-ras \textit{G13D} mutation to occur at far greater incidence in cells derived from xenografted tumors or exposed to conditions of combined hypoxia and hypoglycemia \textit{in vitro}. Interestingly, this mutagenesis was neither enhanced by induced oxidative damage nor prevented by the antioxidant vitamin E. Moreover, the accumulation of K-ras mutations was paralleled by down-regulation of the key mismatch repair protein MSH2 in xenografted tumors, particularly in hypoperfused areas and under hypoglycemic conditions \textit{in vitro}. In contrast, the microsatellite stable colorectal cancer cell line Caco-2 neither accumulated K-ras mutations nor showed down-regulation of MSH2 under these conditions. Thus, our study suggests that ischemia may not simply select for, but can actually trigger, increased mutation rate in crucial colorectal cancer oncogenes. This finding establishes a novel linkage between genetic instability, tumor ischemia, and genetic tumor progression and carries important implications for applying anticancer therapies involving tumor hypoxia (e.g., antiangiogenesis) in microsatellite instability cancers. (Cancer Res 2005; 65(18): 8134-41)

Introduction

The \textit{ras} proto-oncogenes control transduction of signals required for proliferation, differentiation, and survival (1), mainly acting as GDP/GTP-regulated binary switches located at the inner surface of the plasma membrane (2, 3). There is a growing understanding of the complexity of direct and indirect intracellular effectors of Ras, many of which contribute to cellular transformation, including the Raf/KSR/MEK/MAPK, PI3K/AKT, and RalGDS pathways (2). It is also understood that in normal cells, this signaling network contains several self-inhibitory feedback mechanisms that prevent protracted and unscheduled activation (4).

The human genome contains three \textit{ras} genes (K-\textit{ras}, N-\textit{ras}, H-\textit{ras}), each of which can be affected by point mutations in codons 12, 13, and 61, resulting in constitutive activation (locking in the GTP-bound state) of the respective Ras proteins. The resulting continual transmission of Ras-dependent signals serves as a potent oncogenic stimulus and was recently documented to suffice for triggering incipient tumorigenicity in susceptible tissues (5, 6). Mutations of the K-\textit{ras} gene are among the most common genetic alterations in human cancer and occur in ~50% of colorectal cancer, 75% to 100% of pancreatic cancer, and almost 50% of lung adenocarcinomas (1, 7). It is also noteworthy that expression of mutant \textit{ras} genes is often associated with chemoresistance and radioresistance of the respective malignancies (8, 9).

The role of mutant K-\textit{ras} is of considerable interest in the case of colorectal cancer where there is evidence that this genetic lesion is required for aggressive and continuous tumor growth (“tumor maintenance”; ref. 10) and angiogenesis (11). Therefore, it is reasonable to ask: How do K-\textit{ras} mutations emerge in colorectal cancer, at what stage of tumor progression, and why? Sporadic colorectal cancer is thought to progress along two molecularly and clinically distinct pathways. One arises as a result of development of multiple polyps (mostly due to somatic loss of the \textit{APC} gene), later followed by mutation of K-\textit{ras}, and development of mismatch repair–proficient, microsatellite stable, and chromosomally unstable cancer cell phenotype. Colorectal cancer may also originate on the background of mismatch repair deficiency and microsatellite instability and acquire later transforming mutations, including those of the K-\textit{ras} gene (12), although the loss of mismatch repair capability per se may not be the initiating defect in such cases.

In the latter type of colorectal cancer, hereditary nonpolyposis colorectal cancer, key effectors of DNA mismatch repair such as MLH1, MSH2, or PMS1/2 become lost, silenced, or dysfunctional leading to an apparent “mutator phenotype” (13). Normally, MSH2 forms heterodimers with other mismatch repair genes. The MutS\textit{x} complex, composed of MSH2 and MSH6, is responsible...
for recognition and repair of base-to-base mismatches and insertion/deletion loops of up to 8 bp. The MutSβ complex, comprised of MSH2 and MSH3, also repairs insertion and deletion loops but is unable to repair bp mismatches (14). In human colorectal cancer, the predominant K-ras mutations are G→A transitions and G→T transversions, with codons 12 and 13 the most frequently affected (15).

It is of interest that in colorectal cancer, K-ras mutations are often observed at the stage of a relatively large intermediate adenoma (16) and in association with overt three-dimensional growth (17, 18). This circumstance could be associated with a selective growth advantage of cells expressing mutant K-ras under conditions where cell survival may be challenged by anoikis, growth factor deprivation, and decreasing access to the vasculature (all of which may be alleviated by expression of the oncogenic Ras; ref. 19). Alternatively, it is possible that the tumor microenvironment (known to be mutagenic in many systems; refs. 20–22) may somehow trigger or accelerate the rate of K-ras mutations in colorectal cancer.

Here, we show that, indeed, mismatch repair–deficient (but not proficient) colorectal cancer cells manifested an accelerated accumulation of K-ras mutations in vivo and under oxygen/glucose deprivation in vitro, apparently independent of oxidative damage per se. Moreover, this microenvironmental up-regulation of the mutator phenotype was coupled with down-regulation of the MSH2 gene product. We discuss the implications of this quantitative modulation of the mismatch repair deficiency for anticancer therapy of microsatellite instability–type colorectal cancer and cancer in general (e.g., in the context of angio genesis inhibition).

Materials and Methods

**Cell lines.** Human colorectal cancer cell lines DLD-1 and HCT116 both harbor one mutant K-rasG13D allele, and in Dks-8 (from DLD-1) and Hkh-2 (from HCT116), the mutant K-ras allele was selectively disrupted by homologous recombination (10); thus, Dks-8 and Hkh-2 sublines are K-rasG13D/null. Caco-2, which is K-ras wild-type (23), was used as a mismatch repair–competent colorectal cancer cell line, as were normal human dermal fibroblasts.

**In vivo evaluation in human tumor xenograft model.** All in vivo procedures were done according to the guidelines and recommendations of the Canadian Council of Animal Care and approved by the University of Guelph local Animal Care Committee. Colorectal cancer cell lines were used for parallel cell culture and s.c. implantation of 1 × 10^6 to 2 × 10^6 cells into the flank of immunodeficient RAG1 null mice (24). Tumor growth was monitored and tissues were harvested once tumor volume reached ~100 to 300 mm^3. At least three pieces of each tumor were snap frozen in liquid nitrogen for subsequent protein and DNA extraction and analysis. For immunostaining, HCT116 xenografted mice were injected i.p. with 150 mg/kg body weight Hypoxyprobe-1 (Chemicon International, Inc., Temecula, CA) 1 hour before euthanasia. Tumors were then removed and samples for cryosections, protein, and DNA isolation were snap-frozen and stored at −80°C for subsequent analysis.

**In vitro oxygen and/or glucose deprivation.** Cells were maintained in standard culture conditions: DMEM (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum, 50 µg/ml gentamicin, 1 mmol/L sodium pyruvate, and 5 µmol/L of fungizone, at 37°C in a humidified atmosphere containing 5% CO_2_. Glucose deprivation was done by substituting normal DMEM (1000 µg/ml) with glucose-free DMEM (Sigma-Aldrich). Hypoxic conditions were achieved using a Modular Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA) modified to permit continuous flushing of the chamber with a humidified mixture of 95% N_2_ and 5% CO_2_. The oxygen content in the chamber was kept at <0.1% in all hypoxia experiments. Confluent monolayers were trypsinized, and 5 × 10^5 cells were seeded into 100 mm plates, which were incubated under normal cell culture conditions overnight. Thereafter, the plates were assigned to one of four groups—control, hypoglycemia, hypoxia, and hypoglycemia plus hypoxia—and exposed to these conditions for 24 or 48 hours.

**Mismatch repair function.** The effect of tumor microenvironment on mutagenesis was quantified by determining 6-thioguanine resistance (i.e., mutation at the hprt locus) in cultured HCT116 cells (pretumor), in HCT116 cells recovered from xenografts (posttumor), and in HCT116 cells under oxygen and/or glucose deprivation as described above. For mutagenesis, 5 × 10^4 HCT116 cells were plated into 100 mm culture dishes in the presence of 37.5 µg/ml 6-thioguanine and cultured for 14 days. Check plates were established with 100 HCT116 cells and incubated for the same time in the absence of 6-thioguanine. After incubation, plates were rinsed and stained with 0.1% crystal violet in methanol, washed, dried, and colony numbers counted. Mutation frequency was determined as the number of colonies formed in treated plates, normalized for the colony formation efficiency from the check plates. Cells were isolated from xenografts by collagenase digestion and passaged once before 6-thioguanine selection. Values were normalized to control colony formation for each experiment.

**K-rasG13D mutation analysis.** DNA from human dermal fibroblasts or human blood leukocytes was used as wild-type K-ras control, and DNA from HCT116 cells (heterozygous mutant at codon 13; ref. 10) was used as a positive control for codon 13 K-ras mutation. DNA was extracted from cultured cells, xenografted tissues, and blood cells, and K-rasG13D mutations were determined using a PCR/RFLP analysis with the restriction enzyme XcmI. PCR was done in a reaction volume of 50 µl containing 250 µg DNA, reaction buffer, 0.2 mmol/L deoxynucleotide triphosphate, 1.5 mmol/L MgCl_2_, 0.2 µmol/L of each primer, and 2.5 units of Taq polymerase (Invitrogen Canada, Inc., Burlington, ON, Canada). The primer sequences, modified from previously reported ones (25), were RAS C47-C56 (sense) 5’-ACTGAAATATAACCTTGTGGTCCATGGA-3’ and RAS C47-C56 (antisense) 5’-TACTGTATCAAAGAATGGTCCTGCACCAG-3’. Amplification was done by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes.

Ten microliters of the PCR reaction were digested with XcmI (10 units; New England Biolabs, Inc., Beverly, MA) for 20 hours at 37°C in a total volume of 40 µl. When codon 13 is wild-type, the PCR product contains a restriction site for XcmI, and digestion yields bands of 137 and 28 bp. If there is a mutation in either of the first two bases of codon 13, the mutant PCR fragment will not be cut and will remain at its original size of 165 bp. Digested products were visualized by electrophoresis on a 3% agarose gel (containing 0.3 µg/ml ethidium bromide) and densitometric analysis was done using a Fluorchem 8800 system (Alpha Innotech Corp., San Leandro, CA). Calculations were done using a curve generated from measurements of a Low DNA Mass Ladder Standard (Fermentas Life Sciences, Burlington, ON, Canada) and a comparison of the 137 bp (digested wild-type) and 165 bp (undigested mutated) bands, and the ratio of mutant DNA to wild-type DNA was calculated for each sample. To enhance the 165 bp products, which represent the mutated DNA, a second PCR-RFLP using the products of the first digestion as templates was done. The same primers from the first PCR could thus more selectively amplify the undigested mutated DNA from the first digestion. This two-step PCR-RFLP led to selective amplification of the mutated K-ras–containing bands (165 kb) that were excised from the gels, purified using a Qiagen gel Extraction kit (Qiagen, Inc., Mississauga, ON, Canada), and sequenced at the Guelph Molecular Supercentre. Primer Ras C47-C56 was used for this reaction.

**Protein isolation and Western blotting for MSH2.** Cell pellets were washed with PBS and resuspended in 0.5 ml of cold fresh lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 0.5 mmol/L MgCl_2_, 0.2 mmol/L EGTA, and 50 mmol/L Tris-HCl (pH 7.5), with aprotinin (2 µg/ml), DTT (2 mmol/L), and phenylmethylsulfonyl fluoride (1 mmol/L) added just before use]. After vortexing and centrifugation (12,500 × g at 4°C for 10 minutes), the supernatant was aliquoted and stored at −80°C for future use. Pieces of tumor xenografts were lysed in a buffer containing 2% Triton X-100, 0.02% MgCl_2_ in PBS (pH 8.3) as described above. Ten to 50 µg of total protein from
samples were run on a 10% polyacrylamide gel under reducing conditions. Protein was then transferred to polyvinylidene difluoride membrane followed by incubation in 5% milk diluted in 0.1% Tween 20 in TBS. The membrane was then probed for chemiluminescent detection of MSH2 using a mouse primary antibody (1:666; Oncogene Science, Cambridge, MA), and a goat anti-mouse peroxidase-conjugated antibody (1:10,000; Sigma-Aldrich). Blots were normalized using a mouse anti α-tubulin antibody (1:400,000; Sigma-Aldrich) and densitometry was done using AlphaEaseFC software (Alpha Innotech).

Immunostaining. Tumor xenografts were immunostained for MSH2 protein (paraffin and cryosections) and for dual MSH2/Hypoxyprobe-1 (cryosections). For paraffin sections, 5-μm-thick sections were deparaffinized, and EDTA antigen retrieval [1 mmol/L (pH 9.0), 95 °C] was used. Sections were then blocked in 4% normal goat serum and incubated with mouse anti-MSH2 primary antibody (1:75; Oncogene Science) followed by goat anti-mouse IgG, conjugated biotin (1:200) for AEC histochemistry (Sigma-Aldrich), and hematoxylin counterstaining. Five-micrometer-thick cryosections were fixed in 4% paraformaldehyde, blocked in DAKO blocking solution, then incubated with mouse anti-MSH2 primary antibody (1:75; Oncogene Science) followed by goat anti-mouse IgG, conjugated with Cy3 fluorochrome (1:200; Sigma-Aldrich). Anti–Hypoxyprobe-1 antibody (Chemicon International) was directly labeled with Alexafluor488 according to the instructions of the manufacturer (Invitrogen Canada) and used at 1:25 dilution. Images were captured using an Olympus BX61 microscope equipped with appropriate excitation and emission filters.

Oxidative damage. To investigate whether oxidative stress, generated by hypoxia/reoxygenation treatment, plays a role in mutation of the K-ras gene, cultures of Dks-8 and Hkh-2 cells were supplemented by the antioxidant vitamin E (50 μmol/L final concentration) before exposure to hypoxia plus hypoglycemia conditions. After 48-hour exposure, the cells received fresh complete media without vitamin E and were kept in a standard incubator (at 37 °C in a humidified atmosphere containing 5% CO2) for an additional 4 or 48 hours. Additional cultures of Dks-8 and Hkh-2 cells were exposed to the oxidant H2O2 (40 μmol/L final concentration) for 48 hours in a standard incubator. Cells were pelleted, washed with PBS, and proteins and DNA were obtained as described above. Simultaneous control cultures were included in all studies. As a consequence of oxidative damage, carbonyl groups are introduced into protein side chains by a side-specific mechanism. To determine the level of protein oxidation, an oxidized protein detection kit (Oxyblot, Chemicon International) was used. This kit is based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine (DNPH; ref. 26). The samples were treated with DNPH and the amount of carbonyl groups was measured by Western blotting according to the protocol supplied with the kit. Western blots were digitized and quantified by computer-assisted imaging using the Fluorchem 8800 supplied by Alpha Innotech. The relative optical densities of treated samples were compared with those of control samples. Each experiment was done in duplicate and repeated at least twice. K-ras mutation analysis was done on DNA obtained from the same cells to disclose the status of K-ras mutation as described above. Cells exposed to hypoxia and low glucose for similar time points were used as positive controls for K-ras mutation.

Statistical analysis. Quantitative data are presented as means of several independent measurements ± SD. Statistical analysis (t test) was used to determine differences between means within experiments, with a two-tailed level of significance (P < 0.05).

Results

Elevated K-ras mutation in xenografts. The mutant K-ras–positive HCT116 and DLD-1 colorectal cancer cell lines are highly aggressive and capable of forming overt tumors within 10 days after s.c. injection into immunodeficient mice. In contrast, s.c. injection of their K-ras–negative isogenic counterparts Hkh-2 and Dks-8, respectively, either did not lead to any tumor formation (1 × 106 cells) or tumors did appear but after a lengthy latency period of 20 to 30 days or longer (2 × 106 cells; Fig 1A). In the case of an independent K-ras–negative cell line Caco-2, only 40% (4 of 10) of mice injected s.c. with cancer cells (2 × 106) formed tumors, and with a latency of at least 4 months (Fig 1A). Thus, high tumorigenicity cosegregates with the status of mutant K-ras in this panel of colorectal cancer cell lines.

As has been shown in numerous systems, our results with the hprt assay confirm that cells recovered from HCT116 xenografted tumors displayed elevated genetic instability as revealed by enhanced mutation rates compared with starting cell lines (Fig 1B). Interestingly, HCT116 cells exposed in vitro to low glucose,

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or low glucose plus hypoxia, had significantly greater colony numbers after 2 weeks of 6-thioguanine selection than did control cultures, or cells exposed to hypoxia alone \((P < 0.05, \text{Fig. 1B})\). We then further explored the possibility that this increased genetic instability might target the biologically relevant oncogene \(K\text{-ras}\).

Whereas inactivation of mutant \(K\text{-ras}\) in Hkh-2 and Dks-8 cells led to significantly diminished \textit{in vivo} growth capacity (as reported previously in ref. 10), aggressive tumors eventually developed (Fig. 1). This suggests that some of these cells might have acquired secondary genetic alterations that could compensate for the absence of the \(K\text{-ras}\) oncogene. This is made more likely in view of the known microsatellite instability and mismatch repair deficiency of both parental cell lines (HCT116 and DLD-1; refs. 27, 28). Given the central role of Ras pathway activation in colorectal cancer, we reasoned that one target of such secondary mutations in Hhk-2 and Dks-8 cells could have been the remaining \(K\text{-ras}\) allele. As expected, all wild-type and mutant \(K\text{-ras}\) alleles were detected in both parental (HCT116 and DLD-1) cell lines in culture and in their corresponding tumors (Fig. 2A). Interestingly, tumors showed an enrichment for mutation at codon 13, compared with starting cells (Fig. 2A). In contrast, \(K\text{-ras}\) mutations were not detectable in Dks-8 and Hkh-2 cell lines (which were recombined before these experiments to ensure homogeneity), but were clearly present in the corresponding tumors, especially when the two-step PCR-RFLP was used (Fig. 2B). There was no detectable mutation of \(K\text{-ras}\) codon 13 in the microsatellite stable Caco-2 cells and tumors using the two-step PCR-RFLP (Fig. 2B). The detection limit of the two-step PCR-RFLP assay was found to be one in 2 \(\times 10^6\) cells, as determined by serial dilutions of \(K\text{-ras}\) mutant (DLD-1) in wild-type cells (Dks-8; data not shown). Using similar PCR-RFLP approaches (25, 29), we further examined \(K\text{-ras}\) mutations in codons 12 and 61 on the above cell lines and xenografts and found no evidence for \textit{de novo} development of mutation at these additional codons in xenografted tumors (results not shown; Supplementary Data). Taken together, these results suggest that microsatellite instability-type (but not microsatellite stable) colorectal cancer cells develop \textit{de novo} \(K\text{-ras}\) mutations under tumor microenvironment growth conditions.

**Changes in MSH2 expression in colorectal cancer tumors \textit{in vivo}**. Thus, preexisting microsatellite instability background of DLD-1 and HCT116-derived cells results in acquisition of new \(K\text{-ras}\) mutations not constitutively (as might be expected), but only upon exposure to the tumor microenvironment. This suggests existence of cooperating changes in the mismatch repair phenotype of these cells. In this regard, HCT116 and DLD-1 cells are deficient for MLH1 and MSH6, respectively (27, 28); therefore, we decided to test the \textit{in vivo} status of MSH2, another major regulator of mismatch repair. Interestingly, expression of the MSH2 protein was significantly down-regulated in the tumors compared with the corresponding cultures of these cells, albeit differences were noted between individual tumors and between different pieces of the same tumor (Fig. 3A). This heterogeneity of MSH2 staining prompted us to examine the location of MSH2-positive and MSH2-negative HCT116 cells within the tumor mass. Immunohistochemistry revealed a gradient distribution of MSH2 staining within viable regions of HCT116 xenografts (Fig. 3B), whereas entire viable regions of Caco-2 xenografts were consistently stained for this antigen (Fig. 3C). Interestingly, MSH2 expression in HCT116 xenografts manifested an inverse relationship with proximity to the regions of ischemia as indicated by mutually exclusive staining for MSH2 and hypoxic (but still viable) tumor cells (Fig. 3D-F).

**\(K\text{-ras}\) mutations and MSH2 down-regulation as a function of microenvironmental influences**. Exclusion of MSH2 expression from ischemic/hypoxic regions of the tumor mass suggests that microenvironmental influences associated with deficient perfusion may be involved in reducing MSH2 expression and \textit{de novo} \(K\text{-ras}\) mutations in microsatellite instability colorectal cancer cells. In this regard, ischemia is known to result in hypoxic and hypoglycemic conditions and we chose to evaluate these factors as possible inducers of increased \(K\text{-ras}\) mutability. We, therefore, cultured Hkh-2, Dks-8, Caco-2, or normal fibroblasts under hypoxic and hypoglycemic conditions and compared the consequences of these treatments for the \(K\text{-ras}\) status (Fig. 4A). Indeed, microsatellite instability cell lines (Hkh-2 and Dks-8) acquired new \(K\text{-ras}\) mutations under these conditions, whereas their microsatellite stable counterparts (Caco-2, fibroblasts) did not (Fig. 4A).

In agreement with these observations, hypoxia combined with hypoglycemia also led to down-regulation of MSH2 expression in cultured HCT116 and DLD-1 cells, which did not occur in Caco-2 cells (Fig. 4B). Interestingly, hypoxia alone had a minimal effect on expression of this mismatch repair regulator that was only significantly down-regulated by the exposure to hypoglycemia alone or in combination with hypoglycemia in both HCT116 and DLD-1 cells (Fig. 4B), suggesting that changes in perivascular glucose levels provoke quantitative changes in mismatch repair.
deficiency and may contribute to new K-ras mutations in colorectal cancer cells in vivo.

Reactive oxygen species–induced oxidative damage does not affect K-ras mutation. To investigate whether hypoxia reoxygenation–type injury could be the cause of K-ras mutations observed in this study, the effect of the dietary antioxidant vitamin E on the mutation rate was assessed. We found that vitamin E (50 μmol/L) remarkably reduced the amount of oxidized proteins in treated samples compared with controls in cultures exposed to hypoxia plus hypoglycemia. There was a 10-fold (Hkh-2) and 3-fold (Dks-8) decrease in the amount of protein carbonyl groups derivatized with DNPH in vitamin E–treated cultures versus controls (Fig. 5A). Immunohistochemistry staining of xenografted HCT116 cells (B) and Caco-2 cells (C) reveals differential distribution of MSH2 protein in the nuclei of viable clusters of cells (*), necrotic regions typical of xenografts produced by these cell lines. In HCT116 tumors, staining is highly heterogeneous, with regions of normal cellular architecture lacking detectable MSH2 protein (stars in B). In contrast, Caco-2 xenografts show homogenous MSH2 staining throughout viable regions (C). D, immunofluorescence staining of MSH2 protein (red) in an HCT116 xenograft. E, detection of hypoxic areas (green) by anti-Hypoxyprobe-1 in the same field. F, overlay of the two images (D and E) demonstrating an inverse relationship between MSH2 expression and hypoxic regions of the tumor. Areas of necrosis are negative for both MSH2 expression and hypoxic protein adduct formation (*). Bar, 30 μm.

Discussion

Our studies seek to establish the possible linkage between tumor cell access to perfused/functional blood vessels and prevalence of key oncogenic events involved in tumor progression. We focused on expression of activated K-ras in human colorectal cancer cells because this particular event commonly occurs at defined (intermediate) stages of the disease progression, has implications for tumor angiogenesis, and plays a paradigmatic role in the emergence of the transformed cancer cell phenotype (5, 6, 16, 19). In this regard, our study led to several novel findings. First, we detected an increased rate of mutational activation events affecting the K-ras gene (K-ras<sup>G13D</sup>) during growth of colorectal cancer cells as tumors in vivo. Second, this oncogenic trend could be duplicated in vitro, but only under the influence of hypoxia and hypoglycemia. Third, mutations of K-ras occurred only in colorectal cancer cells with preexisting mismatch repair deficiency, but not in their counterparts with microsatellite stable phenotype. Fourth, increased acquisition of K-ras mutations under hypoxic/hypoglycemic conditions (in vitro and in vivo) was paralleled by down-regulation of the MSH2 gene product, an indication of deepening mismatch repair-deficient phenotype of these cells. These observations have implications for the nature of the mutator phenotype.
neither was able to affect K-ras \( G13D \) mutation in colorectal cancer cells. Moreover, exposure of the cells to \( \text{H}_2\text{O}_2 \) did not lead to K-ras \( G13D \) mutation. Therefore, we suggest that K-ras mutation caused by hypoxia and hypoglycemia may occur independently of hypoxia-reoxygenation oxidative damage per se. Hypoxia can also lead to deregulation of several molecular mechanisms involved in maintaining DNA integrity, including functional and expression status of ATM/ATR, p53, rad51, and MSL1 (30, 39). However, as in vivo hypoxia is often inseparable with ischemia, other factors associated with blood supply could be considered as regulators of the mismatch repair phenotype.

In this regard, our study shows that the expression of one of the key mismatch repair genes, MSH2, is affected (down-regulated) by glucose deprivation, and in tumors in vivo. Whereas acidosis, reperfusion, or unspecified "nutrient deprivation" were implicated as cofactors in hypoxia-dependent genetic instability (30), our data indicate that specific microenvironmental factors present in hypoperfused domains of tumor masses (namely hypoglycemia) were necessary and sufficient to cause MSH2 down-regulation in mismatch repair–deficient HCT116 and DLD-1 cells but not in mismatch repair–proficient Caco-2 cells. Recently, hypoxia-related down-regulation of rad51 has also been reported with concomitant disruption of homologous recombination (31), and growth of cancer cells in three-dimensional spheroid culture lead to decreased production of PMS2 and concomitant reduction in DNA mismatch repair capacity (40), suggesting that microenvironmental regulation of DNA repair events may be widespread in tumor progression. In our study, it is also noteworthy that K-ras mutations occurred only in the context of the combined constitutive mismatch repair defect (MLH1 mutation (27) in HCT116 cells and their Hkh-2 derivative; MSH6 loss (28) in DLD-1 cells and their Dks-8 derivative), superimposed with ischemia/hypoglycemia dependent down-regulation of MSH2, but not in mismatch repair–proficient Caco-2 cells. Finally, whereas ras oncogenes themselves have been implicated as causes of genetic instability (41), differential mutant K-ras (transformation) status of HCT116 and Hkh-2 cells did not prevent either of these cell lines from accumulating K-ras mutations. Thus, a multifactorial nature of both mismatch repair defects and microenvironmental conditions seems to be necessary to drive progression of colorectal cancer cells toward increasing malignancy.

While this article was under review, a report was published indicating that hypoxia alone significantly down-regulates MSH2 in cultured HCT116 cells (42), and that this event is dependent on intact tumor suppressor p53. In our study, hypoxia alone did induce a detectable, but slight, reduction in MSH2 protein levels, but this effect was greatly exceeded by the MSH2 down-regulation induced by hypoglycemia, a condition that was not examined by Koshiji et al. Further, we were able to show that MSH2 protein levels are reduced to similar levels in p53 wild-type (HCT116) and p53 null (DLD-1) colorectal cancer cells, suggesting that there may be oxidative stress factors, such as formation of reactive oxygen species or of 8-oxoguanine and thymine glycols (35).

When oxygen is suddenly restored to an oxygen-deprived area, the enzyme xanthine oxidase produces oxygen radicals from the newly supplied oxygen, leading to tissue damage (36). Stress conditions, similar to those that exist in the microenvironment of solid tumors, can enhance genetic instability and are reported to be mutagenic to cultured cell lines (37, 38). However, in our study, vitamin E supplementation greatly inhibited oxidative damage but did not afford any protection against K-ras \( G13D \) mutation in Hkh-2 and Dks-8 cell lines. Moreover, exposure of the cells to \( \text{H}_2\text{O}_2 \) did not lead to K-ras \( G13D \) mutation. Therefore, we suggest that K-ras mutation caused by hypoxia and hypoglycemia may occur independently of hypoxia-reoxygenation oxidative damage per se.

In cancer, mechanisms of genetic tumor progression, and the consequences of anticancer therapies that induce transient or chronic ischemic conditions.

It is widely accepted that cancer cells sustain (and display) an increased rate of genetic defects (i.e., possess a mutator phenotype), either spontaneously derived and/or resulting from various stresses, including directly genotoxic therapies (radiation, chemotherapy; ref. 13). This genetic instability is believed to be a consequence of disequilibrium between the magnitude of mutational insults and the ability of the DNA repair machinery to repair the emerging errors. Indeed, hypoxia has been proposed as a source of genetic instability (20) and shown to affect integrity of the genome at several levels, causing faulty DNA synthesis, nucleotide excision repair and mismatch repair, "replication misfiring" and gene amplification, single- and double-strand DNA breaks, decreased homologous recombination, and several more complex genetic lesions (30, 31). Using Big Blue rat-derived cell lines (32, 33), we recently showed that mutation rate in the \( cII \)-shuttle \( cII \) transgene increased in cells recovered from xenografts, compared with their in vitro counterparts (34). In vitro exposure of these cells to hypoxia also led to enhanced \( cII \) mutagenesis. In both cases, the majority of mutations consisted of base substitutions leading to point mutations (34). The sources of this promutagenic effect of hypoxia are thought to include enhanced DNA damage due to hypoxia/reoxygenation-related
significant interaction between cell stress responses and DNA repair enzyme production.

One important aspect that our study documents is the relationship between ischemic conditions, quantitative increase in mismatch repair deficiency, and emergence of a significant oncogenic event in a specific cancer context. Indeed, mutant K-ras is both frequently expressed (1) and indispensable (10) for malignant progression of colorectal cancer. Whereas this is related to multiple cellular and molecular properties of Ras (2), of particular interest in the context of the present study is the relationship between expression of oncogenic K-ras and induction of tumor angiogenesis (11) and resistance to ischemic conditions (43). In fact, such dual relationship between genetic lesions and both "vascular supply" and "vascular demand" of cancer cells may apply to other oncogenic events as well (44-46). Interestingly, our present study suggests that, in addition to triggering angiogenesis, conditions of vascular deficit and ischemia (glucose deprivation) may generate increased frequency of K-ras mutations, and possibly provide mutant cells with a selective growth advantage (47). This may explain why K-ras mutations usually do not accompany early (small well-vascularized in situ) colorectal lesions, yet become more readily detectable in larger (likely ischemic) tumors (16-18).

The linkage between accumulation of K-ras mutations in colorectal cancer cells and ischemia-like conditions may have important therapeutic implications. In particular, targeting tumor vessels with antiangiogenic and antivascular agents (48) is based on intentional induction of an ischemic state within the tumor, something postulated to subsequently lead to elimination/arrest of large numbers of hypoxia-sensitive cancer cells and ultimately control/cure of the disease (49). Indeed, such an antiangiogenic agent (Avastin) has recently been approved for human use after its action directed at inhibition of the vascular endothelial growth combined with standard chemotherapy led to significant, albeit not curative, responses of patients with advanced colorectal cancer (50). Our study suggests that whereas such therapy may well prove invaluable, additional measures and caution might be needed in the context of tumors with mismatch repair deficiency, especially if the latter is modulated by ischemia. This is because ischemia may trigger additional genetic lesions affecting transforming genes; thus, the mismatch repair status may require some consideration in the context of planned antiangiogenic therapy.

In conclusion, our study shows that human colorectal cancer cells defective in mismatch repair displayed an induction and/or enrichment for the K-ras13D mutation, when exposed to tumor microenvironment conditions (in vitro and in vivo), with a concomitant reduction in expression of the mismatch repair protein MSH2. Thus, this study reveals a significant connection between stressful microenvironmental conditions (hypoxia and especially hypoglycemia), and the generation of mutations in the important human oncogene K-ras. These results show that genetic instability is modulated by the cellular microenvironment, which has significant implications for our understanding of colorectal cancer progression, especially in the context of defective mismatch repair.

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References


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