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Suppression of EthylNitrosourea-induced Schwannoma Development Involves Elimination of neu/erbB-2 Mutant Premalignant Cells in the Resistant BDIV Rat Strain

Andrea Kindler-Röhrborn, André B. Kind, Bernd U. Koelsch, Christine Fischer, and Manfred F. Rajewsky

Institute of Cell Biology (Cancer Research), University of Essen Medical School and West German Cancer Center Essen, D-45122 Essen, Germany [A. K.-R., A. B. K., B. U. K., M. F. R.], and Institute of Human Genetics, University of Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany [C. F.]

Abstract

Contrary to the response of rats of the highly sensitive inbred strain BDIX, BDIV rats are resistant to the induction of malignant schwannomas by exposure to the alkylating N-nitroso carcinogen N-ethyl-N-nitrosourea (EtNU). In BDIX rats, a point mutation at nucleotide 2012 in the transmembrane region of the neu/erbB-2 gene has proved to be a very early marker of initiated Schwann precursor cells with an elevated risk of malignant transformation, and is diagnostic of the resulting schwannomas. To gain insight into the cellular and molecular mechanisms responsible for the resistance of the BDIV strain, comparative quantitative neu mutation analyses combined with histomorphological studies were performed on the trigeminal nerves of EtNU-treated BDIV and BDIX rats as well as on their (BDIX × BDIV) F1 progeny. It was found that neu-mutant Schwann cells are initially present at comparable frequency in the trigeminal nerves of both resistant and sensitive animals. Contrasting with the progressive multiplication of mutant Schwann cells in BDIX trigeminal nerves, however, the numbers of mutant cells began to decrease during the intermediatory phase of the carcinogenic process in BDIV animals, and premalignant neu-mutant cells were no longer detectable by the time BDIX rats developed full-blown trigeminal schwannomas. The resistance of BDIV rats thus involves the elimination of initiated neu-mutant Schwann cells during the postinitiation period of EtNU-induced schwannomagenesis via mechanisms that remain to be clarified.

Introduction

Depending on their genetic background, inbred rodent strains exhibit a broad spectrum of sensitivity toward the induction of malignant tumors by specific carcinogens, ranging from high susceptibility to almost entire resistance (1). Although humans, too, may be intrinsically resistant to carcinogenesis, the contribution to cancer resistance in humans is much more difficult to assess because the individual exposure to carcinogens is usually unknown and multigenic inheritance as well as low penetrance preclude the detection of human gene carriers (1, 2). Nevertheless, at least part of the responsible gene homologues may be identified in rodent models (3). A number of resistance loci for different types of tumors have been mapped in appropriate genetic crosses of mice (1) and rats (4, 5). With a single exception (6), however, none of the genes involved has thus far been identified. In principle, genes conferring differential resistance toward the development of tumors can exert a wide variety of functions and act during different phases of the process of carcinogenesis (7–10). Functional studies on resistance mechanisms may thus lead to a better understanding of the multistep process of carcinogenesis and may facilitate the identification of relevant candidate genes and pathways for cancer preventive intervention.

Rats of the inbred BD strains (11) provide an excellent model for the study of resistance to chemically induced carcinogenesis in the peripheral and central nervous system because these strains exhibit differential sensitivity toward the induction of neural tumors by pre- or perinatal pulse-exposure to EtNU4 (12, 13). Thus, BDIX rats develop malignant schwannomas, predominantly of the trigeminal nerves, with an incidence >85%, whereas BDIV rats are entirely resistant (12, 14). Schwannoma development is strongly suppressed in (BDIX × BDIV) F1 rats, consistent with a dominant resistance gene with decreased penetrance and/or with polygenic inheritance. On the basis of targeted linkage mapping in genetic crosses of BDIX and BDIV rats, we have recently reported a locus on chromosome 10 that is associated with susceptibility/resistance toward schwannoma development (14).

A transversion mutation at nucleotide 2012 in the transmembrane region of the neu/erbB-2 gene is likely to be the initial event in EtNU-induced schwannoma development in sensitive strains of rats (15). This mutation (16) is diagnostic of EtNU-induced rat schwannomas (15, 17) and in the process of oncogenesis characterizes a subset of immature Schwann cells that are mainly located near the nerve-brain junction and exhibit unrestrained proliferative activity in contrast to their differentiating wild-type counterpart cells. neu-mutant Schwann cells are, therefore, at high risk of progressing toward the expression of fully malignant phenotypes. Different from other animal models of strain-specific oncogenesis that rely on the detection of complex histomorphological alterations as premalignant lesions (10, 18), the identification of mutant neu-alleles in the DNA of microdissected trigeminal nerve tissue allows us to reproducibly quantify premalignant cells before any morphological changes become detectable as well as later on during the process of carcinogenesis. Because neu mutation-tagged cells can be followed regardless of alterations in cell morphology or cell migration, the rat model of EtNU-induced carcinogenesis in the trigeminal nerve is particularly well suited to gain insight into potential resistance mechanisms operating in a stage-specific manner.

In the present study, we have quantified the neu-mutant alleles in the trigeminal nerves of resistant BDIV and predominantly resistant (BDIX × BDIV) F1 animals in comparison with the sensitive BDIX rats as a function of time after exposure to EtNU on postnatal day 1 in parallel to histomorphological analyses.

4 The abbreviation used is: EtNU, N-ethyl-N-nitrosourea.
Materials and Methods

Animals and Carcinogen Treatment. Inbred BDIX and BDIV rats (11), as well as first-generation crosses of genders in both orientations ((BDIX × BDIV) F1 and (BDIV × BDIX) F1), were kept under standard conditions in the animal facility of the Institute of Cell Biology (Cancer Research), University of Essen Medical School. Rats of both strains and their F1 progeny received a single s.c. injection of EtNU (80 μg/g body weight) 24 h postnatally (19). Control animals were injected with buffer only.

Preparation of Histological Samples. From postnatal day 30 onward, groups of 3–10 rats were sacrificed with CO2 at defined intervals. The intracranial portions of the trigeminal nerves were dissected out under a stereo operation microscope, snap frozen in liquid nitrogen, and stored at −80°C until cryosectioning (15). In most cases, both nerves were used. Frozen sections (8 μm) were dried and stained with Mayer’s hemalum.

Microdissection. To reproducibly analyze trigeminal nerve tissue near the nerve-brain junction known to contain neu-mutant premalignant cells (15), this area was microdissected from hemalum-stained longitudinal sections of the central nerve region. Adjacent brain tissue and the meninges were removed with a sterile needle under the stereo microscope, and a 1-mm-wide tissue specimen was then transferred into a PCR tube.

Quantitative neu Mutation Analysis in Microdissected Nerve Tissue. Fluorescence-labeled PCR amplification of a 129 bp neu gene fragment from microdissected material was carried out in a total volume of 25 μl. Final concentrations of deoxynucleotide triphosphates were 0.2 mM. Primer 402e was implemented in SAS V.6.12 on PC. Pointwise denaturing 6% polyacrylamide gels [acrylamide: N,N,N,N-tetramethylethylenebisacrylamide 19:1; Ready Mix Gel, A.L.F. grade, Pharmacia Biotech] in TBE buffer on an A.L.F. DNA Sequencer (Pharmacia Biotech).

For each sample, fluorescence-labeled fragments [wild-type allele (129 bp), mutant allele (104 bp)] were quantified by calculating the peak integrals (Fragment Manager program; Pharmacia Biotech), and their ratios were determined.

Statistical Analysis. Each trigeminal nerve was regarded as an independent unit of observation with respect to the fraction of mutant neu alleles. Group comparisons were performed using the nonparametric Wilcoxon test as implemented in SAS V.6.12 on PC. Pointwise P values were reported.

Results

Quantification of Mutant neu Alleles by Fluorescence-labeled PCR Fragment Length Analysis. To comparatively determine the amounts of neu-mutant alleles in both rat strains, length analyses of PCR amplified fluorescence-labeled fragments of the neu gene were performed on an automated DNA sequencer. The characteristic T:A→A:T transversion mutation at nucleotide 1201 of the neu gene was detected by the resulting additional restriction site for MnlI. Calibration was performed by measuring the ratio of wild-type to mutant alleles with an SD of <1% was 5%. Fig. 1 shows examples of fragment length analyses of trigeminal nerve DNA from EtNU-treated and control animals. Mutant neu alleles could be detected from day 30 after EtNU exposure onward.

![Fig. 1. A, B, C, D, E, F.](image)

Retention time (min)

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Fractions of Mutant neu Alleles in Trigeminal Nerve Tissue of BDIX and BDIV Rats and Their F1 Progeny Exposed to EtNU on Postnatal Day 1. Groups of 3–10 BDIV and F1 animals were sacrificed at time intervals of 5 and 10 days between postnatal days 30 and 150 and between postnatal days 30 and 200, respectively, after EtNU-exposure. Thereafter, intervals were extended until day 500. For BDIX rats, fewer time points were chosen because the kinetics of neu-mutant cells had been determined before (15; Fig. 2A). Because neu-mutant cells reside predominantly close to the nerve-brain junction where microtumors arise at later stages (15), this region was microdissected from 188 trigeminal nerves of 101 BDIV rats, from 126 nerves of 76 BDIX rats, from 223 nerves of 119 (BDIX × BDIV) F1 progeny, and from 222 nerves of 119 (BDIV × BDIX) F1 animals treated previously with EtNU on postnatal day 1. Microdissected tissue was used as a source of DNA for the subsequent fluorescence-labeled PCR.

Fig. 2 shows the fractions of neu-mutant alleles in the trigeminal nerves of BDIX, BDIV, and (BDIX × BDIV) F1 progeny, as a function of time after exposure to EtNU. The fractions of neu-mutant alleles represent a measure of the numbers of initiated premalignant cells (see “Discussion”). To better describe and summarize the data obtained, the time axis was operationally divided into three phases. During phase I (days 30–100 after EtNU-exposure; Fig. 2A), the median percentage of mutant alleles was 6.1%, varying between 0 and 26.2%, in the transformation-sensitive BDIX rats. Animals displaying symptoms indicative of trigeminal schwannomas were frequently
found to carry one overt tumor only. Therefore, the amount of premalignant cells present at earlier stages may vary considerably between the two nerves. Schwann cells near the nerve-brain junction of the resistant BDIV rat initially (phase I) displayed mutant alleles at higher rates than the Schwann cells of BDIX rats (median, 12.81%; minimum, 0%; maximum, 52.1%; \( P = 0.0001 \); Fig. 2B). During phase II (100–260 days), the frequency of mutant alleles markedly increased in the trigeminal nerves of EtNU-treated BDIX animals (median, 18.3%; minimum, 0%; maximum, 93.2%; Fig. 2A). However, all of the BDIX rats scheduled to be sacrificed at later time points had to be sacrificed before day 260 because of the presence of trigeminal schwannomas. Tumors, as well as the contralateral nerves in cases where they were intact, were also used for mutation analysis. Contrary to their frequency in BDIX trigeminal nerves, the frequency of neu-mutant alleles continuously decreased in the trigeminal nerves of BDIV rats during phase II and became almost undetectable during phase III (260–500 days), with a median of 2.9, a minimum of 1.1, and a maximum of 27.3 (\( P = 0.0001 \); combined data for phases II and III; Fig. 2B). All of the BDIV rats remained available for analysis without showing any symptoms of trigeminal schwannomas.

In EtNU-treated F1 animals, ~20% of which had been shown previously to exhibit trigeminal tumors (14), a mixed type of kinetics was observed for the frequency of neu-mutant alleles (Fig. 2C). Because no significant difference between rats of both orientations of gender was observed, the data were combined. During phase I, the great majority of animals displayed values similar to those determined for BDIX rats (median, 2.9%; minimum, 0%; maximum, 52%). During phase II, high mutation frequencies were observed in a small subgroup of F1 animals, similar to the values detected previously in BDIX rats. However, most animals displayed low frequencies of mutant alleles during phases II and III (median, 5.1; minimum, 0; maximum, 85.1). None of the F1 animals had to be sacrificed before the time points originally scheduled for analysis.

**Histomorphological Observations.** The nerve-brain junction was examined histomorphologically in trigeminal nerve sections from EtNU-treated BDIX, BDIV, and (BDIX × BDIV) F1 rats, as well as from untreated control animals. Time intervals were the same as those used for mutation analysis, with the earliest time point at 30 days after EtNU-exposure. neu-mutant cells typically appear as multiple groups of irregularly distributed cells with an increased nuclear:cytoplasmic ratio (early atypical proliferates; Refs. 15 and 20).

Histomorphological observations paralleled the findings at the molecular level. As exemplified in Fig. 3, there was no visible difference regarding the frequencies and sizes of early atypical proliferates in the susceptible BDIX and the resistant BDIV rats up to postnatal day 70, whereas untreated control animals neither exhibited morphologically altered cells nor atypical tissue architecture. About 110 days later on, the frequency and size of nests of atypical-looking cells had gradually increased and dominated the nerve-brain junctional area in most of the EtNU-treated BDIX animals. In contrast, the trigeminal nerves of BDIV rats had gradually become devoid of the premalignant cells and at 180 days after EtNU-exposure appeared almost identical to untreated controls. Most trigeminal nerves of F1 animals (data not shown) were histomorphologically indistinguishable from those of their BDIV parents. In a few F1 rats, however, the number of premalignant cells increased during phase II and finally gave rise to full-blown schwannomas.

**Discussion**

Resistance to the development of chemically induced tumors in inbred rodent strains has been observed frequently (1). However, neither the underlying biological mechanisms nor the genes involved have thus far been identified.

Using a combination of molecular and histomorphological analyses, we have attempted to obtain information on the cause of the resistance of BDIV rats to the development of EtNU-induced schwannomas, in contrast to the high susceptibility of the BDIX strain. In BDIX rats, immature trigeminal Schwann precursor cells exhibiting a T:A→A:T transversion at nucleotide 12 of the neu gene transmembrane region are observed from a very early stage of carcinogenesis onward up to the appearance of full-blown malignant schwannomas (15). These cells are arrested in maturation and, contrary to their differentiating neighboring cells, continue multiplying and, therefore, are at high risk of progressing to the expression of fully malignant phenotypes. The fate of these cells can be monitored along the process of carcinogenesis in vivo. Comparative quantitative neu gene mutation analyses of the trigeminal Schwann cell population of BDIV, (BDIX × BDIV) F1, and BDIV rats as a function of time after exposure to EtNU, paralleled by histomorphological analyses, were thus performed in the present study.

Limitations to an absolute proportionality between the amount of neu-mutant alleles and the number of initiated cells are: (a) the distribution of cell cycle phases in the target cell population; and (b) at a later stage of the carcinogenic process, loss of the wild-type neu allele, as detectable from around day 70 after EtNU-exposure onward.

The neu gene mutation at nucleotide 2012 in trigeminal Schwann cells of BDIV rats was first detected on day 30 after carcinogen exposure and further on throughout phase I (days 30–100) of carcinogenesis (see “Results”). Surprisingly, the frequency of mutant neu alleles detected in BDIV trigeminal nerves during phase I was even higher than in the susceptible BDIX animals. This observation ex-
ELIMINATION OF PREMALIGNANT CELLS IN CANCER-RESISTANT RATS

Fig. 3. Morphological appearance of the trigeminal nerves of BDIX and BDIV rats at different times after exposure to EtNU on postnatal day 1 and of age-matched untreated control animals. A–E, frozen sections of the brain (b)-nerve (n) junction stained with H&E. A and B, trigeminal nerve tissue from BDIX rats at 70 and 180 days, respectively, after exposure to EtNU. C and D, trigeminal nerve tissue from BDIV rats, at 70 and 180 days, respectively, after exposure to EtNU. E and F, trigeminal nerve tissue from age-matched untreated BDIX control animals. Note the similarly increased density of nuclei in A and C in comparison with E. B represents a full-blown schwannoma of a susceptible BDIX rat at 180 days. After the same time interval, D resembles the untreated control tissue F.

includes resistance mechanisms based on a strain-specific differential frequency of mutational initiation, caused, e.g., by differential DNA repair capacity. Moreover, a smaller size of the BDIV target cell population, potentially attributable to a different timing of trigeminal development between the two strains, is also unlikely to play a significant role.

The results of the present mutation analyses are mirrored by the histomorphological appearance of BDIV versus BDIX trigeminal nerves during phase I. Around day 70, groups of small round atypical cells that carry the mutant neu gene, as shown previously (15), accumulated near the trigeminal nerve-brain junction in both BDIV and BDIX rats. Both the frequency and size of these lesions were indistinguishable between the two rat strains. The identical histomorphological appearance of BDIV and BDIX trigeminal nerves during the early phase of carcinogenesis is evidence for the fact that the resistance of BDIV rats is effected by mechanisms acting during later steps in the carcinogenic process. Observations in other experimentally induced rodent tumors, e.g., 1,2-dimethylhydrazine-induced mouse colon tumors (7) and rat mammary tumors induced by N-methyl-N-nitrosourea (8, 9), suggest a similar sequence of events.

During phase II (days 100–260) of schwannomagenesis (see “Results”), a striking difference was observed between the trigeminal nerves of the two strains. In EtNU-exposed BDIX rats, the frequency of mutant alleles drastically increased up to peak values of >90%, reflecting the development of full-blown schwannomas with loss of heterozygosity for the wild-type neu allele (clearly confirmed by the histomorphology of BDIV trigeminal nerves on day 180). In contrast, neu-mutant Schwann cells gradually disappeared from the BDIV trigeminal nerves, and the mutation became undetectable after day 220. Accordingly, the histomorphological appearance of BDIV trigeminal nerves on day 180 closely resembled that of untreated control animals.

The resistance of BDIV rats is thus apparently attributable to the elimination of neu-mutant premalignant cells. In most of the (BDIX × BDIV) F1 rats, too, neu-mutant cells disappeared during phases II and III. However, in accordance with the fact that ~20% of EtNU-exposed F1 rats developed trigeminal schwannomas (14), a subgroup of F1 animals exhibited high levels of mutant alleles (up to 80%) in their trigeminal nerves through phase II. Because of, perhaps, a second event during oncogenesis [possibly BDIV allele-specific loss of heterozygosity on chromosome 10 (14)], these neu-mutant cells may thus have been saved from the BDIV-specific control mechanisms.

Various mechanisms could be responsible for the removal of initi-
ated cells. Among these are host factors such as immunosurveillance as well as tissue-specific processes. neum-mutant initiated Schwann cells within the trigeminal nerve could, for example, be removed by cytotoxic T cells or natural killer cells. Recent work by Altschmidt et al. (21) has shown that syngeneically transplanted BDIX schwannoma cells do indeed induce a specific T-cell response; but they simultaneously secrete factors, such as transforming growth factor β (TGF-β), which inhibit T-cell proliferation, thereby enabling schwannoma cells to escape immunosurveillance (21). A differential capability of premalignant Schwann cells to escape immunosurveillance might translate into differential susceptibility.

Alternatively, neum-mutant cells in BDIV trigeminal nerves, contrary to their premalignant counterpart cells in BDIX rats, might be induced to undergo apoptosis at a rate exceeding their proliferation rate and thus ultimately disappear. If initiated BDIV Schwann cells undergo apoptosis at an elevated rate, the molecular stimuli triggering this response at >100 days after carcinogen exposure will have to be identified as well as apoptosis-associated genes that might be functionally polymorphic between BDIV and BDIX rats.

Redifferentiation of glutathione S-transferase 7-7 positive preneoplastic plastic foci induced in the liver of resistant Copenhagen (Cop) rats has been suggested to be a putative resistance mechanism (18, 22). This option can be excluded for the disappearance of premalignant neum-mutant Schwann cells in the trigeminal nerves of BDIV rats. Mutant alleles are no longer detectable at 220 days after carcinogen exposure, even further distal to the nerve-brain junctional area. The premalignant cells must, therefore, have been eliminated from the nerve tissue rather than having migrated out or differentiated. Our ongoing studies are directed toward clarification of the mechanism(s) responsible for the elimination of premalignant Schwann cells by functional and morphological analyses.

A phenomenon similar to the one described here has been observed regarding the resistance of Cop rats to the induction of mammary tumors by N-methyl-N-nitrosourea (10). After exposure to N-methyl-N-nitrosourea, Cop rats developed characteristic preneoplastic lesions after a period of time corresponding to the time seen in the susceptible Wistar Furth (WF) strain. In this model, the cells of many lesions displayed an activating mutation of the Ha-ras gene in both the WF (75%) and the Cop rat (60%). Despite this fact, ras-mutant cells failed to progress toward malignancy and ultimately disappeared in the resistant strain (9, 10). Crosses with immune-deficient nude rats have shown that the resistance of Cop rats does not depend on the presence of T cells (23).

Resistance to cancer development effected by the elimination of premalignant cells at postinitiation stages of carcinogenesis not only calls for clarification of the underlying mechanisms and identification of the responsible genes; it also invites cancer-preventive approaches based on the interference with intermediary stages in the process of carcinogenesis preceding the onset of the growth of malignant tumors.

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References

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