Nitric oxide-derived reactive species have been implicated in many disorders. Protein nitrotyrosine is often used as a stable marker of these reactive species. Using immunohistochemistry, we have previously detected nitrotyrosine in murine Mutatect tumors, where neutrophils are the principal source of nitric oxide. We now report on the identification of several prominent nitrotyrosine-containing proteins. Using Western blot analysis, nitrotyrosine in higher molecular mass proteins (>20 kDa) was detected in tumors containing a high number of neutrophils but not in tumors with fewer neutrophils. Staining for nitrotyrosine was consistently seen in low molecular mass proteins (≤15 kDa), regardless of the level of neutrophils. Protein nitrotyrosine was not seen in Mutatect cells growing in vitro. Treatment with nitric oxide donors produced nitration of ≤15-kDa proteins, but only after extended periods. These small proteins, both from tumors and cultured cells, were identified by mass spectrometry to be histones. Only a subset of tyrosine residues was nitrated. Selective nitration may reflect differential accessibility of different tyrosine residues and the influence of neighboring residues within the nucleosome. The prominence of histone nitration may reflect its relative stability, making this post-translational modification a potentially useful marker of extended exposure of cells or tissues to nitric oxide-derived reactive species.

Selective nitration of histone tyrosine residues in vivo in Mutatect Tumors*

Arsalan S. Haqqani‡§, John F. Kelly†, and H. Chaim Birnboim‡‡

From the ‡Department of Biochemistry, Microbiology and Immunology, University of Ottawa and the Ottawa Regional Cancer Centre, Ottawa, Ontario K1H 1C4, Canada and the §Institute of Biological Sciences, 100 Sussex Drive, Ottawa, Ontario K1A 0R6, Canada

Nitric oxide (NO)1-derived reactive nitrogen oxide species (RNOS) are cytotoxic and mutagenic, and have been implicated in the pathogenesis of several inflammatory disorders (1–9). Inflammatory cells, such as macrophages, monocytes, and neutrophils produce a relatively large amount of RNOS. These reactive species may exert deleterious effects by modifying or damaging various cellular targets including DNA, lipids, and proteins (10–12). One of the targets in proteins is tyrosine (Tyr), which can be converted into a fairly stable end-product, 3-nitrotyrosine (NTyr) (13). The presence of free NTyr amino acids or NTyr-containing proteins in biological samples is used as a molecular marker of RNOS production in a tissue (13). In addition, nitrination of Tyr residues in proteins may alter protein function (4, 14–17), which may have both physiological and pathological significance. The presence of protein NTyr has been reported in inflammatory disorders such as Helicobacter pylori infection, Crohn’s disease, ulcerative colitis, Wegener’s granulomatosis, cystic fibrosis, asthma, obliterator bronchiolitis, and rheumatoid arthritis (2–9). The level of protein NTyr has been found to correlate with the severity of inflammatory diseases (18–20). Protein NTyr has also been detected in some tumors (21–24). A high level of protein NTyr correlates with poor outcome in melanoma patients (25). Although a high level of protein NTyr has been detected in vivo in human or animal diseases, only a limited number of NTyr-containing proteins has been identified (26–34).

Several methods have been employed to detect and identify protein NTyr. Anti-NTyr antibodies permit ready detection by immunohistochemistry, Western blotting, or enzyme-linked immunosorbent assay (35, 36). Typical methods for identification of specific NTyr-containing proteins involve immunoprecipitation of proteins with specific antibodies (26–31, 37–39). Immunoprecipitation has certain limitations; it is limited to proteins for which specific antibodies are available and it cannot identify which specific Tyr residue(s) have been nitrated. Mass spectrometry of tryptic peptides is a sensitive and specific technique to identify proteins. Recently, it has been shown capable of identifying tryptic peptides containing NTyr and also specifying which Tyr residue(s) has been nitrated. One recent report describes a technique of localizing nitratated proteins by Western blotting on a two-dimensional gel, followed by mass spectrometry, to identify putative nitrated proteins in tissue samples (41). However, positive identification of specific tyrosine residues nitrated in vivo has not previously been reported.

The Mutatect mouse tumor model is a series of mouse fibrosarcoma-derived cell lines that have been engineered to express human interleukin-8 in a regulatable fashion (42). Cells are injected into syngeneic mice where they grow as subcutaneous tumors. Mutatect tumors are infiltrated with inflammatory cells, predominantly neutrophils (43). The number of neutrophils correlates with the number of mutations arising in the tumor cells (42, 43). We have recently shown by immunohistochemistry that tumor-infiltrating neutrophils express inducible nitric-oxide synthase and that protein NTyr is present in these experimental tumors (43). In the present report, we detect NTyr-containing proteins in Mutatect tumors using West-
ern blot analysis, but also use mass spectrometric analysis to unambiguously identify some prominent Tyr residues, including localization of specific Tyr residues that have been nitrated.

**EXPERIMENTAL PROCEDURES**

**Mutatect Cell Culture and Tumor Formation**—Mutatect cells were originally derived from a subcutaneously growing fibrosarcoma that had been induced by methylcholanthrene in a C57BL mouse (44). The properties of Mutatect TM-28 and TM-34 cells, engineered to secrete the neutrophil chemoattractant interleukin-8, have been described elsewhere (45). Cell cultures were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum (Invitrogen, Burlington, Canada) in a humidified atmosphere of 5% CO2, 95% O2 at 37°C. Mutatect tumors were formed by subcutaneous injection of TM-28 or TM-34 cells into syngeneic, 8–10-week-old C57BL/6 J male mice and excised after 3 weeks. Other details have been described earlier (42). All animal experiments were carried out at the Animal Resources facility of the Institute for Biological Sciences, National Research Council in Ottawa in accordance with guidelines of the Canadian Council on Animal Care.

**Exposure of Cells to NO Generating Compounds**—The NO donors used were: (i) methylglyoxal trinitrate (David Bull Laboratories, Canada) or sodium nitroprusside (SNP, Sigma-Aldrich Chemicals, St. Louis, MO), since they have been previously found to be mutagenic toward Mutatect cells (45). 2 × 106 cells were seeded in 10-cm dishes; after cultures were established, the NO-donating drug was added. Exposure was limited to 24 h, 0.5 mM methylglyoxal trinitrate or 1.0 mM SNP was used. At this time of exposure and at these concentrations, the drugs caused 30% cytotoxicity, as determined by trypan blue staining. For longer-term exposures (1–14 days), SNP was used at a lower concentration, 0.1 mM. Culture medium and drug were replaced and cells were subcultured every 3 days. At this concentration, SNP caused only about a 10% reduction in the rate of cell growth. Before harvesting the cells for protein analysis, the plates were washed to remove any detached cells, and the adherent viable cells were harvested by trypsin treatment. Harvested cells were >95% viable as determined by trypan blue exclusion at all times shown. These treated cells and corresponding control cultures were used for protein analysis.

**Nitrite Measurements**—The flux of NO generated in the culture media of Mutatect cells from SNP was estimated by the measurement of the accumulated nitrite levels using the Griess reagent, as described earlier (45).

**Protein Extraction and Western Blot Analysis**—Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.0005% bromphenol blue, 125 mM MOPS, pH 6.8) and boiled for 5–10 min. For extraction of nuclei, cells were swelled in nuclei extraction buffer (10 mM Tris-HCl (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin), incubated on ice for 20 min, and then homogenized with a tight-fitting Dounce homogenizer. Nuclei were collected by centrifugation at 400 × g, resuspended in SDS sample buffer, sonicated, and heated at 100°C for 5–10 min. Protein from tumors was isolated by homogenization and then sonication (in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin), the homogenate was diluted in SDS sample buffer and heated at 100°C for 5–10 min. Fluorescence was used to quantify protein (46). NTyr-containing bovine serum albumin (BSA) was prepared by incubating BSA (6 mg/ml) in 10 mM NaNO2, 9 µM FeCl3, 0.3% H2O2, and 20 mM sodium acetate (pH 5.6) for 24 h at room temperature. All protein extracts were resolved on a 12% discontinuous SDS-PAGE and either stained with Coomassie Brilliant R-250 (Sigma-Aldrich) or electrochemically transferred onto polyvinylidene difluoride membranes (Millipore, Nepean, Canada) using an improved transfer procedure.2 Membranes were stained with Ponceau S (Sigma-Aldrich) to detect the transferred proteins and the molecular weight standards. Where indicated, membranes were incubated with 20 µM Na2S2O4 to chemically reduce NTyr residues (NO2-Tyr) to NH2-Tyr (1). Membranes were washed (10 mM Tris-HCl, 0.1% Tween 20, pH 8), and then blocked with 2% BSA for 30–45 min. Anti-NTyr rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) was diluted in 1:2000 in TBST and incubated with the membranes for 1 h. Membranes were washed 4 times in TBST and then incubated for 1 h with secondary antibody (alkaline phosphatase-linked goat anti-rabbit IgG; Kirkgaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in TBST. Membranes were washed 4 times with TBST and then developed by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich).

**Measurement of Neutrophil Content and hprt Mutants in Tumors**—The neutrophil content in tumors was estimated by quantifying the level of myeloperoxidase, a neutrophil-specific marker. Details of extraction of myeloperoxidase from tumor fractions have been previously described (42). An assay specific for myeloperoxidase was used (47). The frequency of hypoxanthine phosphoribosyltransferase (hprt) mutants was measured in cells growing ex vivo as described previously (42).

**Mass Spectrometric Analysis of Proteins**—Protein extracts were first resolved by SDS-PAGE (20 µg/lane) and silver stained as previously described (42). The protein bands of 15 kDa were excised and in gel-digested with modified trypsin (Promega, Madison, WI) without reduction/alkylation. The digested peptides were first analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager Elite STR (Applied Biosystems, Framingham, MA). The peptide mass fingerprints were used to search a non-redundant protein sequence database (NCBI) using MassFt (Protein ProspectorTM). Tryptic digests of ≤15 kDa proteins from SNP-exposed Mutatect nuclear extracts were analyzed by capillary liquid chromatography-tandem mass spectrometry (CapLC-MS/MS) using a hybrid quadrupole time of flight mass spectrometer (Q-TOFMS, MicroMass, Manchester, UK). The protein extracts were resuspended in 15 µl 0.1% H2O2, 0.1 mM. Culture medium and drug were replaced and cells were incubated on ice for 20 min, and then homogenized with a tight-fitting Dounce homogenizer. Nuclei were collected by centrifugation at 400 × g, resuspended in SDS sample buffer, sonicated, and heated at 100°C for 5–10 min. Protein from tumors was isolated by homogenization and then sonication (in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin); the homogenate was diluted in SDS sample buffer and heated at 100°C for 5–10 min. Fluorescence was used to quantify protein (46). NTyr-containing bovine serum albumin (BSA) was prepared by incubating BSA (6 mg/ml) in 10 mM NaNO2, 9 µM FeCl3, 0.3% H2O2, and 20 mM sodium acetate (pH 5.6) for 24 h at room temperature. All protein extracts were resolved on a 12% discontinuous SDS-PAGE and either stained with Coomassie Brilliant R-250 (Sigma-Aldrich) or electrochemically transferred onto polyvinylidene difluoride membranes (Millipore, Nepean, Canada) using an improved transfer procedure.2 Membranes were stained with Ponceau S (Sigma-Aldrich) to detect the transferred proteins and the molecular weight standards. Where indicated, membranes were incubated with 20 µM Na2S2O4 to chemically reduce NTyr residues (NO2-Tyr) to NH2-Tyr (1). Membranes were washed (10 mM Tris-HCl, 0.1% Tween 20, pH 8), and then blocked with 2% BSA for 30–45 min. Anti-NTyr rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) was diluted in 1:2000 in TBST and incubated with the membranes for 1 h. Membranes were washed 4 times in TBST and then incubated for 1 h with secondary antibody (alkaline phosphatase-linked goat anti-rabbit IgG; Kirkgaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in TBST. Membranes were washed 4 times with TBST and then developed by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich).

**RESULTS**

**Detection of Nitrated Proteins by Western Blot Analysis in Mutatect Tumor Extracts**—We have previously reported on the presence of protein NTyr in Mutatect tumors by immunohistochemical analysis using an anti-NTyr antibody. Both cytoplasmic and nuclear staining in tumor cells was observed (43). To examine in more detail the nature of the NTyr-containing proteins, Mutatect tumor homogenates were analyzed by Western blot analysis using an anti-NTyr antibody. A number of nitrated proteins, ranging in size from large to small, were detected (Fig. 1A). The distribution of NTyr-containing proteins was similar, but not identical, to the distribution of protein detected by Coomassie Blue staining. This suggested that some proteins were preferentially modified. The most prominent examples were ≤15 kDa proteins, presumed to be histones. These results were compared with extracts of cultured Mutatect cells. The difference between tumor extracts and cultured cell extracts was striking (Fig. 1A). In cultured cell extracts, there was only weak staining, limited to proteins of high molecular mass (≥50 kDa); no nitrination of ≤15 kDa protein sequence was detectable. The majority of staining lacking anti-NTyr antibody on Western blots were demonstrated by (i) comparing chemically nitrated and unmodified bovine albumin (Fig. 1B, lanes 1 and 2); (ii) absence of staining with secondary antibody alone (data not shown); and (iii) absence of staining after chemical reduction with Na2S2O4 of NTyr to aminoxyrosine residues (Fig. 1C, NTyr panel). The chemical reduction did not affect the antigenicity of an unrelated protein (thymi-
Selective Nitration of Histone Tyrosines in Mutatect Tumors

FIG. 1. Detection of protein NTyr in Mutatect tumors by Western blot analysis using a specific anti-NTyr antibody. A, Coomassie Blue staining (CB) or Western blotting (NTyr) of protein extracts (30 μg/lane) from either Mutatect TM-28 tumors or cultured TM-28 cells. B, Western blot analysis for NTyr and the subsequent Ponceau S staining of a blot containing 0.5 μg of BSA (lane 1), 0.5 μg of NTyr-containing BSA (lane 2), or 30 μg of protein extract from Mutatect TM-28 tumors (lane 3). Lane 4 is a HeLa extract (20 μg protein) probed with an anti-thymidylate synthase antibody (hTS-8.3) (49). C, same as in panel B, except that all the blots were treated with Na₂S₂O₄ prior to immunoblotting. Other details are described under “Experimental Procedures.”

found in TM-34 tumors from animals receiving no tetracycline (bars 7–11, Fig. 2A). This difference in neutrophils, resulting from the down-regulation of interleukin-8 by tetracycline, was associated with a similar difference in the number of hprt– mutants in the same two groups. The average number of mutants was 450-fold higher in the untreated compared with the tetracycline-treated tumor group (Fig. 2B; note the use of a log-scale). To provide further evidence that neutrophil infiltration into tumors is associated with an increase in RNOS, we performed Western blot analysis on tumor extracts using an anti-NTyr antibody. The number of NTyr-containing proteins differed greatly in tumor samples with a high neutrophil content compared with samples with a low content (Fig. 2C). Tumors with low neutrophil content had a low number of NTyr-containing proteins, which were predominantly ≤15 kDa (Fig. 2C, lanes 1–6); nitration of protein bands at 32 and 34 kDa, consistent with positions for mouse histone H1, were also detected in some tumors. Conversely, tumors with high neutrophil content had a very high number of NTyr-containing proteins, which included proteins of both high and low molecular masses (Fig. 2C, lanes 7–11). These results are consistent with the notion that tumor-infiltrating neutrophils are the principal source of RNOS in Mutatect tumors.

Nitration of Cellular Proteins by Exposure of Cultured Cells to NO Donating Drugs—The data of Fig. 1A indicate that Mutatect cells, cultured under standard conditions, contain a relatively low level of nitrated proteins. Because a high number of nitrated proteins could be detected in Mutatect tumors but not in cultures (Fig. 1A), we tested whether NTyr could be formed after exposure of cultured cells to a NO donating drug. Mutatect TM-28 cells were cultured in the presence of SNP and cell lysates were subsequently analyzed by Western blotting using the anti-NTyr antibody. Exposure for 24 h to 1.0 mM SNP led to nitration of >40-kDa proteins (Fig. 3A). Similar results were obtained after exposing cells for 24 h to 0.5 mM glyceryl trinitrate (data not shown). However, in contrast to tumor extracts (Figs. 1 and 2), ≤15 kDa proteins were not nitrated. We therefore examined the possibility that a longer in vitro exposure (>24 h) to the drugs might mimic in vivo conditions, where cells are grown as subcutaneous tumors for 3 weeks. Cultured cells were therefore exposed for 0, 1, 3, 6, or 14 days to SNP. The concentration was reduced to 0.1 mM, a concentration with the number of mutations arising at the hprt locus (42, 43).

Protein NTyr, Neutrophil Content, and Mutations in Mutatect Tumors—We have previously established that tumor-infiltrating neutrophils are the primary source of NO in Mutatect tumors and that the number of neutrophils correlates with the number of mutations arising at the hprt locus (42, 43). The level of neutrophil infiltration into Mutatect TM-34 tumors can be regulated by the administration of tetracycline, since TM-34 cells express a neutrophil chemokine (interleukin-8) from a tetracycline-responsive (“Tet-off”) promoter (42). The neutrophil content (as measured by myeloperoxidase, a neutrophil-specific marker) varied 16-fold as function of tetracycline added to the drinking water of tumor-bearing mice (Fig. 2A).

Tumors from tetracycline-treated animals (lanes 1–6, Fig. 2A) had an average neutrophil content that was about 5% the level of accumulated nitrite generated from 0.1 mM SNP was about 8.3) (49). Protein NTyr, Neutrophil Content, and Mutations in Mutatect Tumors with low neutrophil content had a low number of NTyr-containing proteins, which were predominantly ≤15 kDa (Fig. 2C, lanes 1–6); nitration of protein bands at 32 and 34 kDa, consistent with positions for mouse histone H1, were also detected in some tumors. Conversely, tumors with high neutrophil content had a very high number of NTyr-containing proteins, which included proteins of both high and low molecular masses (Fig. 2C, lanes 7–11). These results are consistent with the notion that tumor-infiltrating neutrophils are the principal source of RNOS in Mutatect tumors.

Identification of ≤15-kDa Proteins and Sites of Modification—Strong nitration of ≤15 kDa proteins was observed by Western blotting both in Mutatect tumors (Figs. 1 and 2C) and in cells exposed to NO-donating drugs for ≥3 days (Fig. 3C). To identify these proteins, a proteomics approach was used. Proteins from nuclei of Mutatect cell exposed to SNP for 14 days (Fig. 3C) were separated by SDS-PAGE and the 4 discrete ≤15
kDa proteins bands (Fig. 3D) were individually excised. Each was tryptic-digested and the resulting peptides analyzed by MALDI-TOF MS. The majority of tryptic peptides were products of core histones (H4, H2A, H2B, and H4) (data not shown), confirming that the ≤15 kDa proteins were predominantly histones. Once this was established, these peptides were analyzed by LC-MS/MS to determine whether any contained NTyr residues. At least 4 histone-derived peptides were found to contain NTyr residues (Table I). In all cases, the non-nitrated counterpart of each nitrated peptide could also be identified (data not shown). Modification of Tyr to NTyr was found only at specific sites: positions 72 and 98 in H4, position 41 in H3, and...
Selective Nitration of Histone Tyrosines in Mutatect Tumors

Mass spectrometric determination of histone modifications in Mutatect cells exposed to 0.1 mM sodium nitroprusside for 14 days

Nuclear extracts of the cells were run on SDS-PAGE (12%), detected by silver stain and the bands shown in Fig. 3D were excised out and analyzed by CapLC-MS/MS after trypsin digestion.

| Histones | Sequence of the modified peptide | Modification | Site | M<sub>0</sub> | Type |
|----------|----------------------------------|--------------|------|------------|------|
| H4       | TG<sup>C</sup>YFGG<sup>102</sup> | Tyr<sup>D</sup> | 25   | 45         | −NO<sub>2</sub> |
|          | D<sup>28</sup>AVTTEHAK<sup>77</sup> | Tyr<sup>D</sup> | 36   | 45         | −NO<sub>2</sub> |
|          | D<sup>28</sup>AVTTEHAK<sup>77</sup> | His<sup>33</sup> | 45   | 30         | −NO    |
|          | T<sup>C</sup>VAMDVVYALK<sup>31</sup> | Met<sup>83</sup> | 45   | 32         | −O<sub>2</sub> |
| H3       | Y<sup>Y</sup>1RPGTVALKR<sup>48</sup> | Tyr<sup>D</sup> | 36   | 45         | −NO<sub>2</sub> |
|          | F<sup>SC</sup>QSSAMFLQACEAYLVGLFDNTNLCAHAI<sup>112</sup> | Cys<sup>110</sup> | 45   | 48         | −O<sub>3</sub> |
|          | F<sup>SC</sup>QSSAMFLQACEAYLVGLFDNTNLCAHAI<sup>112</sup> | Met<sup>80</sup> or Cys<sup>96</sup> | 45   | 64         | −O and<br>−O<sub>3</sub> |
| H2B      | E<sup>25</sup>SYSVYYVR<sup>43</sup> | Tyr<sup>D</sup>, Tyr<sup>42</sup><br>Tyr<sup>42</sup>/Met<sup>62</sup> | 45   | 45         | −NO<sub>2</sub> |
|          | A<sup>30</sup>MGI<sup>2</sup>MNSFVDIFER<sup>72</sup> | Met<sup>80</sup> and Met<sup>62</sup> | 45   | 64         | −O<sub>2</sub> |
|          | A<sup>30</sup>MGI<sup>2</sup>MNSFVDIFER<sup>72</sup> | Met<sup>80</sup> and Met<sup>62</sup> | 45   | 32         | −O     |
| H2A      | V<sup>V</sup>GAAGPVYMAVLYELTAEILELAGNAAR<sup>71</sup> | Met<sup>71</sup> | 45   | 32         | −O<sub>2</sub> |
|          | V<sup>V</sup>GAAGPVYMAVLYELTAEILELAGNAAR<sup>71</sup> | Met<sup>71</sup> | 45   | 16         | −O     |

<sup>a</sup> The identified sequences correspond to a mouse H4 sequence (NCB accession number S03426), a mouse H3 isotype sequence (H3.1 with NCB accession number S06755), 2 possible mouse H2B isotype sequences (H2B.291A with NCB accession number P10853 or H2B.291B with NCB accession number P10854) and 3 possible mouse H2A isotype sequences (isotype H2A.O with NCB accession number P20670, isotype H2A.B with NCB accession number PP14916 or isotype H2A.B with NCB accession number PP14916). The first methionine of each histone is considered position # 0.

<sup>b</sup> Calculated by subtracting the expected M<sub>0</sub> of the ion from the observed M<sub>0</sub>.

<sup>c</sup> The −O<sub>2</sub> on either Met<sup>80</sup> or Cys<sup>96</sup> and the −O<sub>3</sub> is present on Cys<sup>110</sup>.

<sup>d</sup> Partial nitration of Tyr<sup>37</sup>, Tyr<sup>40</sup>, and Tyr<sup>42</sup>.

positions 37, 40, and 42 in H2B. Other types of protein modification were also detected. Nitrosylation of a histidine residue was observed in H4. Extensive oxidation of methionine and cysteine residues was observed in H4, H3, H2B, and H2A (Table I). These results indicate that exposure of cultured cells to NO-donating drugs can produce extensive modification of histones.

We also examined ±15 kDa proteins present in tumor extracts (Fig. 2C). Extracts were separated by SDS-PAGE and the ±15 kDa protein bands were analyzed as described above. These bands contained tryptic peptides from all 4 core histones (data not shown). The major modification identified in these tumor cell proteins was nitration of Tyr residues at position 96 in H4 (Fig. 4) and position 42 in H2B (data not shown). Mass spectrometry has allowed us to positively identify that the majority of the ±15 kDa proteins are histones. In addition, it has confirmed that NTyr is present in histones derived from both Mutatect tumors and cultured cells exposed to NO-donating drugs. For the first time in an in vivo model, we have shown that Tyr modification was limited to specific sites on a protein.

DISCUSSION

Tyrosine nitration is a covalent post-translational protein modification that occurs widely in association with chronic or recurrent inflammatory diseases (1–9, 18–20) as well as in some tumors (21–25). The consequence of NTyr formation to the disease processes has not been clearly established. However, when known proteins are chemically nitrated, this usually results in interference with their normal function (4, 14–17, 27) and an increase in proteasome-mediated degradation (50). NTyr-containing proteins have been identified in various human and animal diseased tissues (Table II). In most cases, nitrated proteins were identified by a combination of an anti-NTyr antibody and an available protein-specific antibody. In a few cases, N-terminal microsequencing was used to identify the nitrated protein (26, 34). In the case of Mutatect tumors, the abundance and size of four low molecular mass (±15 kDa) proteins in nuclei led us to suspect that they were histones.

Positive identification of all 4 core histones was possible using a combination of mass spectrometric techniques. These techniques have been useful in the past for studying pure proteins that have been chemically nitrated (14, 40, 51), but we have here used these techniques to identify nitrated proteins in complex protein mixtures from tissue samples. This powerful methodology will no doubt see increasing application in the future for the identification of nitrated proteins.

The Mutatect tumor model has been previously useful for establishing a correlation between the number of tumor-infiltrating neutrophils and the number of mutated tumor cells. By using Mutatect cells that express a regulatable form of interleukin-8, we have been able to regulate the number of neutrophils that infiltrate into Mutatect tumors (42). Protein NTyr has been shown earlier by immunohistochemistry in Mutatect tumors (43). Since tumor-infiltrating neutrophils express inducible nitric-oxide synthase and NADPH oxidase, sources of RNOS, these reactive species are likely responsible both for tyrosine nitration and also the observed mutations. In the present report, we have used Western blotting to confirm that Mutatect tumors contain nitrated proteins; by contrast, cultured Mutatect cells contain almost none. The total level of protein nitration was higher in tumors with a high neutrophil content. As expected from earlier studies, the number of neutrophils also correlated well with the number of mutated tumor cells. By establishing a correlation between the number of tumor-infiltrating neutrophils and the number of mutated tumor cells, these results are consistent with the notion that tumor-infiltrating neutrophils are the principal source of mutagenic RNOS in Mutatect tumors.

Nuclear staining for NTyr by immunohistochemistry is often observed in cells in tissues and in NO-exposed cultures (1, 43, 52). Behar-Cohen et al. (52) earlier postulated that histones

3 J. K. Sandhu, S. J. Robertson, H. C. Birnboim, and R. Goldstein, submitted for publication.
might be a target of RNOS in the nucleus. Our finding that histones are the most prominent nitrated proteins in the nucleus provides a possible explanation for the nuclear staining seen by others. Although chemical nitration of histones has been previously demonstrated in vitro (53–55), we report for the first time the prominent nitration of histones in vivo, i.e. both in cultured cells exposed to NO-donating drugs for 3 days and in Mutatect tumor tissues. In NO-exposed cultured cells, nitration of histones was not apparent until 3 days of exposure and then it increased with time, reaching a maximum at about 6 days. Interestingly, in the same cultures, nitration of many cellular proteins >20 kDa was apparent after only 1 day of exposure and increased little with time (>6 days). These >20 kDa non-histone proteins were mainly cytoplasmic proteins. We speculate that the delay in histone nitration may be due to relative inaccessibility of nuclei to the nitrating RNOS. Histones were similarly heavily nitrated in extracts of tumors both with a high and a low neutrophil content. The main difference between the two is that the >20-kDa non-histone proteins were very heavily nitrated in the former but not in the latter. Histones are appreciably more stable than the average cellular protein and their slower turnover may permit them to accumulate NTyr more than high turnover proteins. Thus, the presence of nitrated histones in tissues is potentially a useful marker of long-term exposure to RNOS.

Selectivity of nitration of Tyr residues has been previously studied in known proteins that were chemically modified by nitrating agents (56). These workers suggest that factors favoring Tyr nitration include (i) accessibility of the Tyr residue to nitrating agents; (ii) presence of the Tyr residue in a loop structure formed by amino acid residues Gly or Pro; and (iii) presence of the Tyr in proximity to a negatively charged amino acid residue. In our study, nitration was restricted to Tyr37, Tyr40, and Tyr42 in histone H2B, Tyr72 and Tyr98 in histone H4.
and Tyr\textsuperscript{41} in histone H3. The nucleosome structure shown in Fig. 5 highlights those Tyr residues that are nitrated and those that are not. Of all Tyr residues, Tyr\textsuperscript{42} of H2B is the most accessible to the solvent (as determined using Swiss-PdbViewer software). This site was nitrated both in cultured cells exposed to NO donors and in Mutatect tumors. Nitration of other Tyr residues, such as Tyr\textsuperscript{98} (H4) and Tyr\textsuperscript{111} (H3), may be facilitated by the fact that they are located in close proximity (3–7 Å) to negatively charged amino acids Asp\textsuperscript{J} (H4), Asp\textsuperscript{K} (H2B), and Glu\textsuperscript{L} (H2B), respectively. Nitration of residues Tyr\textsuperscript{40} (H2B), Tyr\textsuperscript{45} (H2B), and Tyr\textsuperscript{111} (H3) may be favored by their proximity to the negatively charged phosphate backbone of DNA in the nucleosome. It has also been postulated that the presence of Cys or Met in the vicinity of Tyr may eliminate interaction of Tyr residues with the nitrating agents, since Cys and Met represent alternative targets for the nitrating agent (56). The absence of nitration of Tyr\textsuperscript{37} (H4), Tyr\textsuperscript{98} (H3), and Tyr\textsuperscript{110} (H2A) may be attributable to the fact that they are in proximity to a Cys or Met; this is supported by the observation that these Cys or Met were identified as being oxidized (Table I). Thus, our observations support the notion that a combination of factors such as accessibility of Tyr residues to the nitrating species, their position in the secondary structure (e.g., loop), and their proximities to Cys, Met, or negatively charged molecules (e.g., Asp, Glu, DNA) may be responsible for the restricted nitration of Tyr sites in histones. In conclusion, mass spectrometric techniques have allowed us to add NTyr to the list of known post-translational modifications of histones that can occur in vivo. The fact that only a limited number of the Tyr residues were nitrated in histones may be due to a combination of factors, including the primary, secondary, and tertiary structure of the nucleosome particle. The demonstration that nuclear proteins (which are in close proximity to DNA) can be nitrated is consistent with our suggestion that RNOS mediates mutations. Since NTyr can readily be detected immunohistochemically, nitrated histones may prove to be useful as a marker of extended exposure of cells or tissues to RNOS.

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SELECTIVE NITRATION OF HISTONE TYROSINES IN MUTATECT TUMORS
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