entiation (van Belzen et al., 1997; Piquemal et al., 1999; Shimono et al., 1999; Gomez-Casero et al., 2001), stress responses (Kokame et al., 1996; Xu et al., 1999; Agarwala et al., 2000; Salnikow et al., 2000; Segawa et al., 2002), and hormone responses (Lin and Chang, 1997; Ulrix et al., 1999; Segawa et al., 2002). Of particular interest is the observation that NDRG1 may have a complex but important function in carcinogenesis (Guan et al., 2000; Salnikow et al., 2000; Gomez-Casero et al., 2001; Nishie et al., 2001; Segawa et al., 2002; Bandyopadhyay et al., 2003) and atherogenesis (Kokame et al., 1996; Sato et al., 1998). Furthermore, attention has been drawn to the fact that this gene was identified as a gene responsible for hereditary motor and sensory neuropathy-Lom (HMSNL) (Kalaydjieva et al., 2000), which is an autosomal recessive form of Charcot-Marie-Tooth disease (CMT) and an early-onset peripheral neuropathy that progresses to severe disability in adulthood. However, the precise role of NDRG1 in the peripheral nervous system (PNS) remains to be elucidated.

It is well known that after axotomy the PNS has the capacity to be repaired by the established sequential process of Wallerian degeneration and ensuing regeneration (for review, see Hirata and Kawabuchi, 2002). Our previous studies (Hirata et al., 1999; Hirata et al., 2000, 2003) obtained stable and consistent results for the time course of the cellular and molecular events seen in the distal stump of the sciatic nerves following crush injury. It is expected that this crush injury model will provide useful clues for exploring the role of NDRG1 in the PNS. In the present study, polyclonal antibody (pAb) was raised against NDRG1 and was used for immunofluorescent labeling of mouse sciatic nerves after crush injury. The results showed that the expression of NDRG1, which was localized in the cytoplasm of Schwann cells (SCs) in intact nerves, dramatically changed during the process of regeneration. The role of the NDRG1 in the PNS is discussed.

MATERIALS AND METHODS

Production of Polyclonal Anti-NDRG1 Antisera

Synthetic peptides corresponding to internal sequences of human NDRG1 were prepared and used as immunogens. These included the TSEGTRSRSC sequence that corresponds to the tandem repetitive region unique to NDRG1 (Kokame et al., 1996; Okuda and Kondoh, 1999; Shimono et al., 1999). The peptides were coupled with keyhole limpet hemocyanin (KLH) and were used to immunize rabbits.

Immunoblotting Analysis

The sciatic nerves of the intact side and the proximal and distal stumps of the operated side at 9 days after the crush injury were homogenized in 500 ml of 1 mM $NaHCO_3$ buffer (pH 7.2) and centrifuged at 9,000 *g* for 15 min at 4°C. The supernatants were subjected to SDS-PAGE and immunoblotting analysis as described previously (Yamanaka et al., 1997), using a pAb to NDRG1 and peroxidase-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA) diluted at 1:1,000 and 1:10,000, respectively.

TABLE 1. Other Primary Antibodies Used in Immunohistochemical Procedures

Antibody (clone)	Structure/ cell recognized	Source	Dilution	Species
MBP	Myelin	Chemicon	1:100	Rat
NF (NE14)	Axons	Boehringer	1:20	Mouse
S-100	Schwann cells	Bio Makor	1:1000	Mouse
BrdU (biotinylated)	Proliferating cells	Oncogene	Ready-to-use	Mouse
S-100	Schwann cells	Nichirei	1:10	Rabbit

Surgical Procedures

Adult male mice (C57BL6) weighing 20–25 g were used for all experiments. The left sciatic nerve was crushed for 30 s with jeweler's forceps at the mid-thigh level under pentobarbital anesthesia. After the surgery, kanamycin sulfate was sprayed over the entire surgical area and the wound was sutured. The intact contralateral side served as a control. Three mice were sacrificed on each of days 1, 2, 3, 7, 9, 14, and 21 after the operation.

The animals were anesthetized with ether, followed by intracardiac perfusions with 0.01 M phosphate-buffered saline (PBS) and then 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The sciatic nerves were removed at a length of about 6 mm, and consisted of the proximal part (2-mm length), the crush injury site (1-mm length) and the distal part (3-mm length). The segments were postfixed with the same fixative for 3 h and immersed in 15% sucrose in 0.1M PB. They were then embedded in Embedding Matrix and immediately frozen with dry ice and isopentane. Longitudinal and transverse serial sections (10-μm thickness) were cut by using a cryostat microtome.

Immunohistochemistry

The immunohistochemical procedure used in the present study has been described elsewhere (Hirata et al., 2003). Briefly, cryostat sections were fixed again with 100% methanol for 10 min at −20°C and then washed with PBS. Nonspecific binding sites were blocked by preincubation with 1% bovine serum albumin (BSA) or 10% Block Ace (Yukijirushi, Sapporo, Japan) in PBS for 1 h at room temperature (RT). For NDRG1 immunohistochemistry, sections were first incubated with a pAb to NDRG1 diluted 1:100 in PBS overnight at RT and then with fluorescein isothiocya-

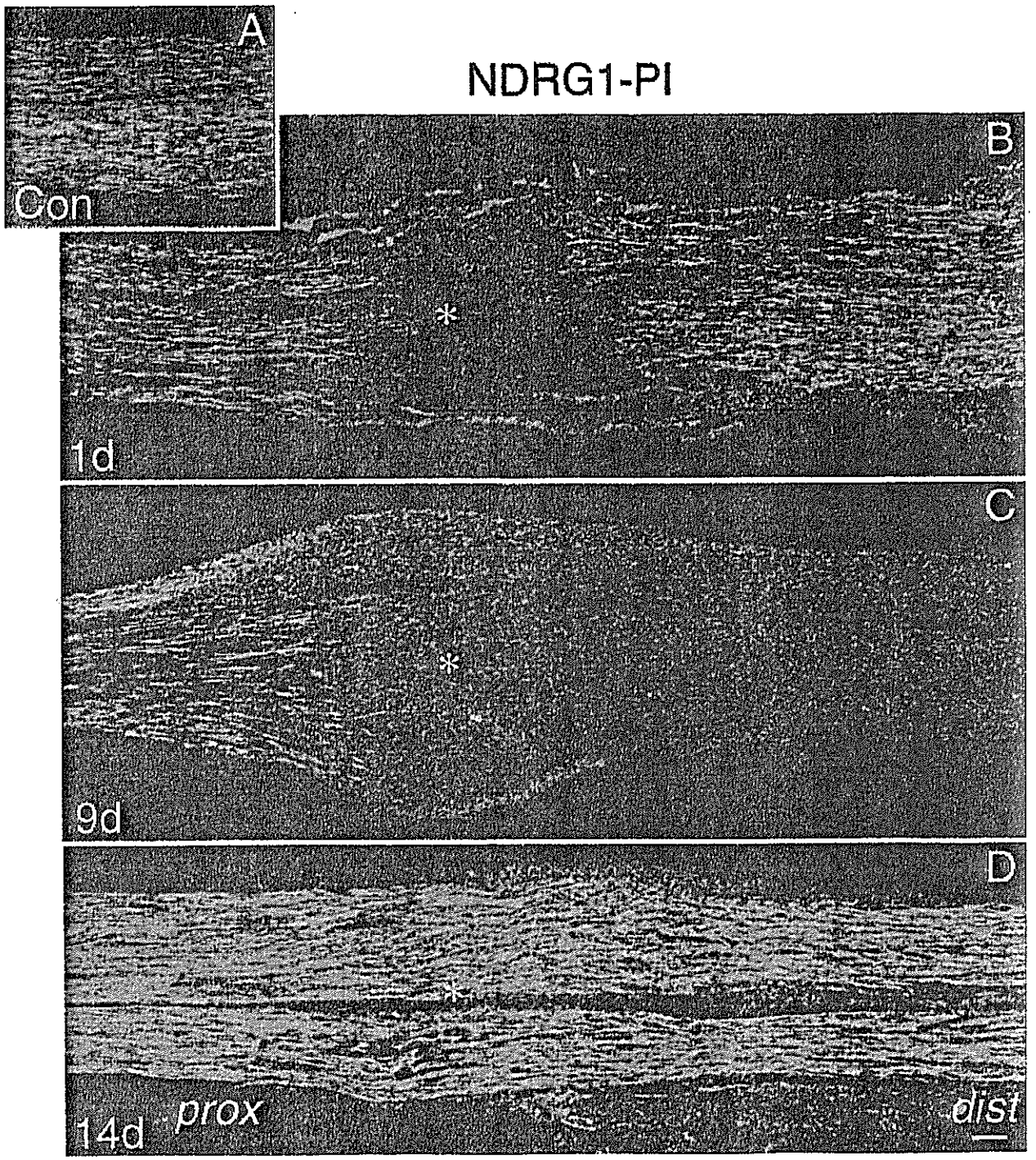

Fig. 1. Expression of NDRG1 (green) in longitudinal sections of intact sciatic nerve used as a control (A) and sciatic nerves at 1 day (B), 9 days (C), and 14 days (D) after crush injury. Moderate NDRG1-immunoreactive (-ir) structures seen in the intact nerve (A) remain in the distal stump at 1 day (B), but are hardly detected there at 9 days (C). The NDRG1-ir structures reappear with stronger immunoreactivity than that in the intact nerves at 14 days (D). Asterisks show the crush injury site. The distal and proximal stumps are indicated by dist and prox, respectively. Red indicates nuclei stained by PI. Scale bar = 50 μm

nate (FITC)-conjugated horse anti-rabbit IgG (Vector, Burlingame, CA) for 4 h at room temperature (RT). Control sections were processed identically and in parallel, except that they were incubated with PBS instead of the pAb to NDRG1. No stained cells were seen in these controls. To identify the nuclei of the cells, the

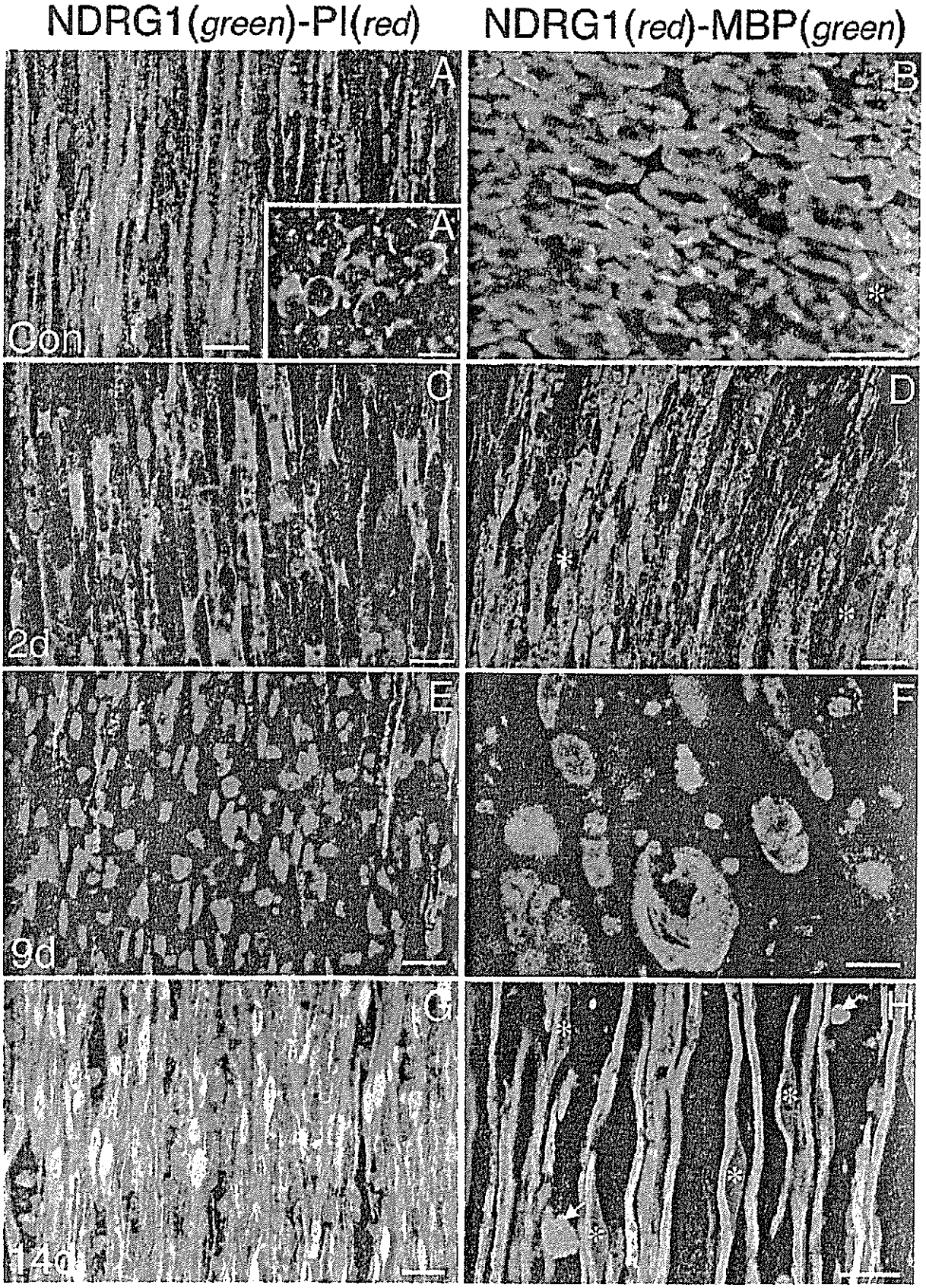
NDRG1(green)-PI(red)
NDRG1(red)-MBP(green)
A
A'
B
Con
C
D
2d
E
F
9d
G
H
14d

Figure 2.

sections were counterstained with propidium iodide (PI) by using a Vectashield mounting medium containing PI (Vector).

To understand the time course of Wallerian degeneration and ensuing regeneration, double immunofluorescent labeling of NDRG1 with a myelin marker (Table 1) was performed. A mixture of a rabbit pAb to NDRG1 and a rat monoclonal antibody (mAb) to myelin basic protein (MBP) was used as the primary antibody. Then, a mixture of Texas red-conjugated donkey anti-rabbit IgG (Jackson) and FITC-conjugated donkey anti-rat IgG (Jackson) was used as the secondary antibody.

Furthermore, double immunofluorescent labeling of NDRG1 with axonal or SC markers (Table 1) was performed. A mixture of a rabbit pAb to NDRG1 and either a mouse mAb to 200-kDa neurofilament protein (NF) or to a mouse mAb to S-100 protein (S-100) was used as the primary antibody. Then, a mixture of FITC-conjugated horse anti-mouse IgG (Vector) and Texas red-conjugated donkey anti-rabbit IgG (Jackson) was used as the secondary antibody. No difference in morphology was noted in any of the immunolabeled structures between the single and double labeling.

To identify mitotic activity of SCs, double immunofluorescent labeling of bromodeoxyuridine (BrdU) and S-100 (Table 1) was performed. Mice were injected intraperitoneally with BrdU (Zymed, CA) (1 ml/100 g) 2 h or 2.5 h prior to being sacrificed. The nerve sections were incubated with a pAb to S-100 as a primary antibody and visualized by Texas red-conjugated donkey anti-rabbit IgG. For subsequent BrdU labeling, part of a BrdU staining kit (Oncogene Research, MA) was used; after treatment with HCl, the biotinylated mouse mAb to BrdU was used as primary antibody. The BrdU binding sites were visualized with streptavidin-FITC (Vector). Double immunofluorescent labeling of NDRG1 and BrdU was attempted, but was unsuccessful since the anti-NDRG1 staining was not compatible with the HCl treatment required for BrdU detection.

Confocal Laser Scanning Microscopy

The sections double-labeled with FITC and PI or FITC and Texas red were scanned with a confocal laser scanning imaging (CLSM) system (LSM-GB200, Olympus, Japan) using excitations at 488 nm (argon laser) for FITC and 568 nm (krypton laser) for PI or Texas red. Single optical sections for each fluorescence were taken separately (channel 1 and channel 2) to avoid any cross-talk and then superimposed. The images were taken using a ×10, ×20, ×40, or ×60 objective lens.

These experiments were reviewed by the Committee on Ethics for Animal Experiments of the Faculty of Medicine, Kyushu University and carried out according to the Guidelines for Animal Experiments of the University, and Law No. 105 and Notification No. 6 of the Japanese government.

RESULTS

In intact sciatic nerves, NDRG1 was expressed over the nerve (Fig. 1A). At 1 day after the crush injury, the expression showed no change except for its disappearance at the injury site (Fig. 1B). At 7–9 days after the crush injury, drastic depletion of the NDRG1 expression was seen at the injury site and the distal stump (Fig. 1C). The expression of NDRG1 then recovered at the injury site and the distal stump at 14 days after the crush injury, showing a slight increase in the immunoreactivity compared to that in the intact nerve (Fig. 1D). Higher magnification of longitudinal (Fig. 2A) and transverse (Fig. 2A′) sections of the intact nerve double-stained with PI nuclear staining showed that NDRG1 immunoreactivity was usually localized in the perinuclear cytoplasm of presumptive SCs of each fiber. Double immunofluorescent labeling with MBP revealed that MBP-immunoreactive (-ir) myelin sheath was usually surrounded by NDRG1-ir cytoplasm (Fig. 2B). At 2 days, cells in the distal stump transformed into cells containing various size of vacuoles, which are known to be characteristic for myelin-phagocytosing cells (Fig. 2C) and double labeling with MBP showed that MBP-ir degraded myelin structures were contained in the vacuoles of NDRG1-ir cells (Fig. 2D). At 7–9 days, NDRG1-ir cells were hardly detected in the injury site (Fig. 2E) and distal stumps (Fig. 2F), where the clearance of myelin debris was considerably progressed (Fig. 2F). At 2 weeks, numerous NDRG1-ir cells with a regular profile similar to that in intact nerves reappeared and the immunoreactivity was stronger than that in the intact nerve (Fig. 2G), and double labeling with MBP revealed that NDRG1-ir cells were in the process of myelinating (Fig. 2H).

Fig. 2. Pseudocolor images of double labeling of NDRG1 (green) with PI (red) (left panel: **A,A′,C,E,G**) and of double immunofluorescent labeling of NDRG1 (red) and MBP (green) (right panel: **B,D,F,H**) of intact nerves (A,A′,B), and of the injury site (E) and the distal stump (C,D,F–H) of crush-injured nerves. All pictures are from longitudinal sections, except for A′,B, which are from transverse sections. Asterisks (B,D,H) indicate sites of nuclei. **A,A′,B:** In intact nerves NDRG1 immunoreactivity is detectable in the perinuclear region of cytoplasm of the presumptive Schwann cells (green in A,A′). **B:** NDRG1-ir cells are myelinating cells, in which MBP-ir myelin sheath (green) are surrounded by a thin layer of NDRG1-ir cytoplasm (red). **C,D:** At 2 days, NDRG1-ir cells (green in C, red in D) have transformed into myelin-phagocytosing cells. D: The vacuoles of the cells seen in C are occupied by MBP-ir degraded myelin structures (green).**E,F:** At 9 days, no clearly stained NDRG1 -ir cells are seen both in the injury site (E) and the distal stump (F). E: Small number of NDRG1 cells seen at the boundary toward the proximal stump (upper). Note that only MBP-ir myelin debris can be seen (green in F). **G,H:** At 14 days, the NDRG1-ir cells reappear showing immunoreactivity stronger (G) than that in the intact nerve (A). H: NDRG1-ir cells are just forming a myelin sheath, judging from the prominent profiles of the perinuclear region (red) and thin MBP-ir myelin sheath (green). Note that strong NDRG1 immunoreactivity is seen not only in the perinuclear region also in the nuclear sites of some cells, suggesting translocation of the protein into nucleus. Arrows indicate the myelin debris. Scale bar = 10 μm.

To identify NDRG1-ir elements, double immunofluorescent labeling with NF, a neuronal marker, or S-100, an SC marker, was carried out. In the cross section of the intact nerve, the NDRG1-ir structure could clearly be distinguished from the NF-ir axons, since the former was localized in the peripheral region of a nerve fiber so that it appeared as a ring-like structure and the latter was in the central part (Fig. 3A). In contrast, the immunoreactive sites of NDRG1 were almost identical with those of S-100 in the intact nerve, suggesting that the NDRG1 was contained only in SCs (Fig. 3B,C). NDRG1 continued to be expressed in S-100-ir SCs, which had the characteristics of myelin-phagocytosing cells at 2 days after the crush injury (Fig.3D,E). At the next stage (7–9 days), the expression of NDRG1 was markedly reduced, whereas the S-100-ir SCs were further transformed to cells with irregular contour (Fig. 3F,G). At the ensuing stage (2 weeks), NDRG1 was reexpressed in S-100-ir SCs, which now showed a regular structure similar to that in the intact nerves.

In the process of nerve regeneration, SCs are known to proliferate following transformation into myelin-phagocytosing cells, and then acquire the immature phenotype to promote axonal regrowth (for review, see Hirata and Kawabuchi, 2002). Double immunofluorescent labeling of BrdU and S-100 was carried out to identify the mitotic activity of SCs. In intact nerves, no cells with BrdU-labeled nuclei were found. At 2 days, only a few S-100-ir SCs with BrdU-labeled nuclei appeared, although presumptive macrophages with BrdU-labeled nuclei were often observed in the perineurium or the epineurium. At 7–9 days (Fig. 4A,C), when the transient depletion of NDRG1 expression occurred, numerous SCs with BrdU-labeled nuclei were detected and distributed throughout the injury site and the distal stump. At 2 weeks, the number of cells with BrdU-labeled nuclei markedly decreased. Thus, the results suggested an inverse relationship between the proliferative activity and NDRG1 expression of SCs. The specificity of BrdU labeling was confirmed in the small intestine (Fig. 4B), where the cells with BrdU-labeled nuclei were specifically distributed in the base of the crypts, known to be the proliferative zone (Potten et al., 1997).

Immunoblotting analysis of sciatic nerves at 9 days after the crush injury revealed that the pAb to NDRG1 labeled a polypeptide band of 43 kDa in the intact nerve (Fig. 5B, lane 1) and the proximal stumps (Fig. 5B, lane 2) but not the distal stumps (Fig. 5B, lane 3) of the crushed nerve. This finding not only confirmed the specificity of the antibody but also indicated preferential depletion of NDRG1 molecules in the injured nerves at this time point. No specific labeling was observed in the control probed with normal rabbit sera.

DISCUSSION

In the present study, immunofluorescent histochemistry and Western blotting analysis using NDRG1 antibodies demonstrated that NDRG1 was expressed in intact mouse sciatic nerve. Double immunofluorescent labeling with an SC marker (S-100) or an axonal marker (NF) showed that NDRG1 was localized in the cytoplasm of SCs, but not in the axons. These findings agree with those of human peripheral nerves analyzed by Northern blotting and RT-PCR (Kalaydjieva et al., 2000) and an immunoenzyme-histochemical technique (Lachat et al., 2002).

Double immunofluorescent labeling of NDRG1 and MBP revealed that NDRG1 was expressed in the cytoplasm of myelinating SCs in intact nerves. Crushed nerves showed little alteration of NDRG1 expression when the myelinating SCs transformed into myelin-phagocytosing cells in the early stage of myelin degradation (Stoll et al., 1989; Hirata et al., 1999). This finding implies that the expression of this protein in SCs is not influenced by either the loss of axonal contact or the transformation of SCs into myelin-phagocytosing cells. Thus, NDRG1 expression does not appear to be regulated during Wallerian degeneration. In contrast, the expression of this protein changed markedly during subsequent process. Both immunohistochemistry and Western blotting analysis demonstrated that NDRG1 expression was dramatically depleted at 7–9 days after the operation, when the myelin removal was considerably progressed. NDRG1 was then reexpressed with more immunoreactivity than that of the intact nerves during remyelination. Thus, our study suggests that NDRG1 may be regulated in the regeneration process of injured nerves.

It is well known that, after taking part in the early stage of myelin removal, SCs proliferate to acquire the immature phenotype and prepare the environments through which regenerating axons grow (for review, see Fawcett and Keynes, 1990; Jessen and Mirsky, 1999; Hirata and Kawabuchi, 2002). In the present study, frequent occurrences of BrdU-labeled SCs were seen at 7–9 days after the crush injury, the timing of which is largely consistent with the occurrence of SC mitosis observed by electron microscopy (O'Daly and Imaeda, 1967). It is noteworthy that the drastic depletion of NDRG1 occurred simultaneously during this period. NDRG1 was reported to be developmentally regulated in embryonic tissues and to be augmented concomitantly with the occurrence of terminal differentiation (Shimono et al., 1999). Direct subtraction of whole mouse embryo cDNAs between the wild type and an N-myc mutant (Shimono et al., 1999) revealed that the NDRG1 gene was repressed by N-myc, a member of the myc family that encodes nuclear phosphoproteins and is believed to play a role in the control of cellular proliferation and differentiation (Melhem et al., 1992). Several lines of in vitro evidence have suggested a role for NDRG1 in cells undergoing terminal differentiation. For example, the NDRG1 gene was upregulated during the differentiation of colon carcinoma cell lines cultured in low glucose medium (van Belzen et al., 1997) and during in vitro forskolin-induced differentiation of a model of the human trophoblast, the chorio-

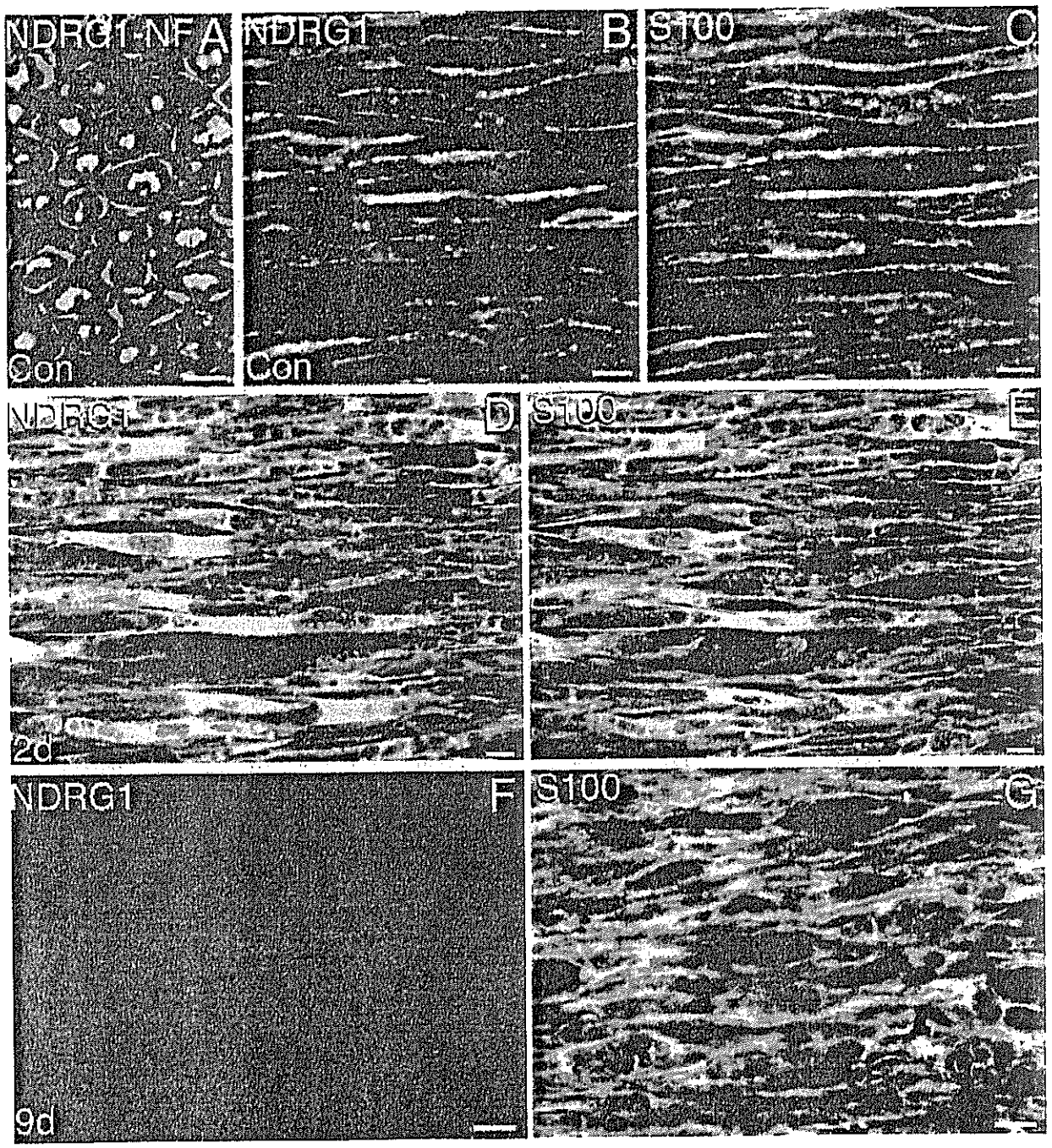

Fig. 3. Gray scale image of double immunofluorescent labeling of NDRG1 and NF in a transverse section of an intact nerve (A) and grayscale images of NDRG1 (B,D,F) and S-100 (C,E,G), in which each of the images is separately displayed, in longitudinal sections of an intact nerve (B,C) and the distal stumps of nerves at 2 days (D,E) and 9 days (F,G) after crush injury. **A–C:** In intact nerves the distribution of NDRG1 (gray in A) and NF immunoreactivity (white in A) is clearly distinguished, since the former is seen in the periphery (cf. Fig. 2A') of the round profile of each fiber and the latter is in the center. On the other hand, the NDRG1-ir sites (B) are almost identical to the S-100-ir sites (C), suggesting that NDRG1 is localized in the cytoplasm of Schwann cells, but not in axons. **D–G:** At 2 days, the expression of NDRG1 (D) is maintained in the S-100-ir Schwann cells which have transformed into myelin-phagocytosing cells (E), but at 9 days, the expression (F) is hardly detected in S-100-ir Schwann cells with a irregular profile (G). Scale bar = 10 μm.

carcinoma BeWo (Xu et al., 1999). The expression of NDRG1 protein was upregulated in the macrophage differentiation of leukemic U937 cells induced by treatment with 1,25-$(OH)_2$ vitamin D_3 or retinoic acid (Piquemal et al., 1999). Furthermore, stable transfection of a colon cancer cell line with NDRG1 cDNA

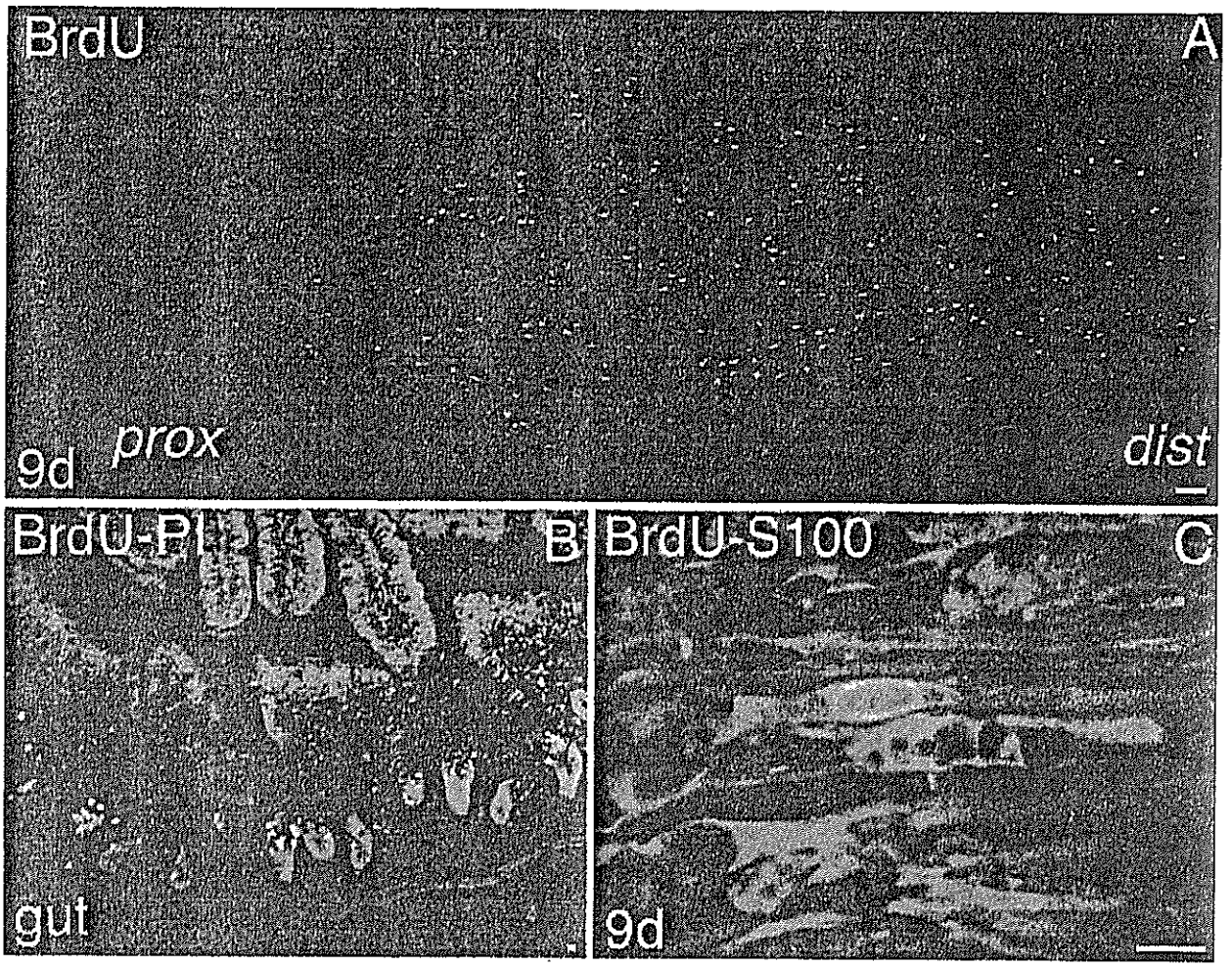

Fig. 4. BrdU immunofluorescent labeling of the nerve at 9 days after crush injury (A,C) and normal mouse jejunum as a positive control (B). **A:** In a longitudinal section of the nerve BrdU-labeled cells (white) are only distributed in the distal stump and the injury site, corresponding to the area where a marked depletion of NDRG1 is seen (cf. Fig. 1C). **B:** In a transverse section of the gut BrdU-labeled epithelial cells (white) are only located in the base of the crypts, to which cell proliferation is known to be restricted. Gray indicates PI-stained structures. **C:** In a higher-magnification image of the distal stump the double immunofluorescent labeling of BrdU (white) and S-100 (gray) shows that the nucleus of an S-100-ir cell is positive for BrdU, suggesting the mitotic activity of Schwann cells. Scale bars = 50 μm in A; 10 μm in B,C.

induced morphological and phenotypic changes indicative of differentiation, suggesting its possible role as a metastatic suppresser gene (Guan et al., 2000). Kurdistani et al. (1998) demonstrated that this gene was a p53-responsive gene with anti-proliferative properties, and that it was regulated in a cell cycle-dependent manner. These in vitro findings were supported by in vivo observations using in situ hybridization or immunohistochemical techniques showing that NDRG1 was expressed in the terminally differentiated cells in some organs, in which cell renewal is detectable under physiological conditions, such as colon epithelial cells (van Belzen et al., 1997), and skin keratinocytes (Gomez-Casero et al., 2001). Our immunohistochemical study on injured sciatic nerves revealed that the expression of NDRG1 was depleted in de-differentiated SCs and recovered in re–differentiated SCs with more immunoreactivity than that in the SCs of intact nerves. Thus, our findings suggest a role for NDRG1 in the terminal differentiation, including myelination, of SCs in nerve regeneration.

In a patient with HMSNL in whom a premature termination codon of the NDRG1 gene was found, one of the main neuropathological features of the disease was SC dysfunction, such as hypomyelination and demyelination/remyelination, failure of compaction of the innermost myelin lamellae, and poor hypertrophic response to the demyelination process (Kalaydjieva et al., 2000). It seems reasonable to surmise that this may be a direct effect of NDRG1 dysfunction, because this protein resides in SCs, especially myelinating ones. Kalaydjieva et al. (2000) inferred that the putative phosphopantetheine-binding domain present in NDRG1 protein (Kokame et al., 1996) may possibly be involved

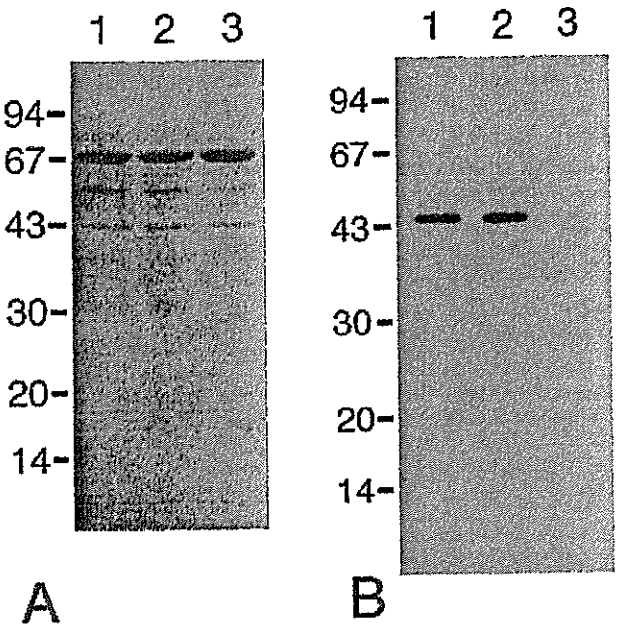

Fig. 5. Immunoblotting analysis of mouse sciatic nerves with a rabbit pAb to NDRG1. The samples were obtained at 9 days after crush injury. The amido black-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles (A) and immunoblotted samples (B) from the intact side (lane 1), and the proximal stump (lane 2) and distal stump (lane 3) of the operated side are shown. The molecular weight markers (kDa) are indicated on the left.

in the lipid biosynthetic pathways operating in the myelinating SCs. On the other hand, Lachat et al. (2002) pointed out that the decompaction of the myelin sheath in the disease may be due to dysfunction of the adherens junctions in Schmidt-Lanterman incisures (Colman et al., 2001) based on their immunohistochemical findings that in all the examined epithelial cell types membrane labeling was observed predominantly adjacent to adherens junctions. Another prominent neuropathological feature of HMSNL is axonal involvement, such as early, severe, and progressive axonal loss (Kalaydjieva et al., 2000). Kalaydjieva et al. (2000) interpreted that NDRG1 may also have a role in the PNS, possibly in the SC signaling necessary for axonal survival, from their assumption that NDRG1 is possibly involved in SC differentiation, since differentiating SCs are an important source of signals for the development of nerves, in addition to controlling neuronal survival (Jessen and Mirsky, 1999). Our findings for NDRG1 expression in the process of nerve regeneration appear to support their hypothesis. However, further study is needed to clarify the exact role of NDRG1 in nerve development, since recent studies have stressed that the developmental process of the nerves is not always identical to the regenerative process, especially regarding the molecular mechanisms regulating SC proliferation (Kim et al., 2000; Atanasoski et al., 2001).

Besides the involvement of the NDRG1 in cell differentiation, this gene has been reported to be modulated under conditions of cellular stress that were caused by homocysteine (Kokame et al., 1996; Agarwala et al., 2000), forskolin (Xu et al., 1999), androgen (Segawa et al., 2002), nickel exposure (Salnikow et al., 2000), and hypoxia (Salnikow et al., 2000; Lachat et al., 2002). Our previous studies demonstrated that two different kinds of heat shock proteins (HSPs), 32 kDa-heat shock protein (Hirata et al., 2000) and 27 kDa-heat shock protein (Hirata et al., 2003) were differentially induced in different phases of SCs after axotomy, since the former was induced in SCs that transformed into myelin-phagocytosing cells immediately after the injury, while the latter was induced in SCs in the next phase that formed the SC column for axonal guidance. In the present study, NDRG1 was reexpressed during remyelination with more immunoreactivity than the normal level, suggesting its involvement in myelination, which is the last phase of SC transformation. No previous investigators have reported that the NDRG1 belongs to the HSPs. However, it is likely that, in addition to HSP32 and HSP27, this protein could be acting as a molecular base, explaining the inherent ability of SCs to not only protect themselves from abnormal environments but also participate actively in the repair process, although it may play a role in maintaining tissue homeostasis in intact nerves.

ACKNOWLEDGMENTS

The authors thank Mr. Yasuhiro Hirakawa and Mr. Takaaki Kanemaru (Morphology Core, Graduate School of Medical Sciences, Kyushu University) for their help in preparing the photomicrographs, and Mr. Naoya Inakura for his expert technical assistance.

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Minireview

The role of nuclear Y-box binding protein 1 as a global marker in drug resistance

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Abstract

Gene expression can be regulated by nuclear factors at the transcriptional level. Many such factors regulate *MDR1* gene expression, but what are the sequence elements and transcription factors that control the basal and inducible expression of this gene? The general principles through which transcription factors participate in drug resistance are now beginning to be understood. Here, we review the factors involved in the transcriptional regulation of the *MDR1* gene. In particular, we focus on the transcription factor Y-box binding protein 1 and discuss the possible links between Y-box binding protein 1 expression and drug resistance in cancer, which are mediated by the transmembrane P-glycoprotein or non-P-glycoprotein. [Mol Cancer Ther 2004;3(11):1485–92]

Introduction

Drug export from cells is mediated through a group of proteins belonging to the ATP binding cassette family of transporters. The 170-kDa transmembrane protein P-glycoprotein (PGP), which is encoded by the multidrug resistance 1 (*MDR1*) gene, is a representative example of an ATP binding cassette transporter. PGP consists of two membrane-spanning domains and two nucleotide binding domains and has been reported to affect the pharmacokinetics of drugs by limiting the rate at which they are absorbed (1–5). Various molecules are targeted by drug treatments for cancer; however, PGP expression is responsible for resistance to the widest range of anticancer drugs (6, 7).

The expression of *MDR1*/PGP in human malignant cancers is expected to play a critical role in limiting their sensitivity to anticancer agents. Therefore, the determination of *MDR1* gene expression levels, along with studies of the regulatory mechanisms of this gene, will be useful in developing tailor-made therapeutic strategies for cancer patients.

The partial sequence of the human *MDR1* gene was first reported in the 1980s (8), and its complete sequence, including clustered CpG sites that are not associated with a TATA box, is now known (9). Within the *MDR1* promoter sequence, a GC box forming a Sp1 site and an inverted CCAAT (ATTGG) site for Y-box binding protein 1 (YB-1) or nuclear factor Y (NF-Y) binding both play key roles in *MDR1* gene expression (10).

MDR1 gene expression is often observed in recurrent cancers and appears after the chemotherapeutic treatment of various human malignancies. In cultured human cancer cells, the *MDR1* promoter was activated by both PGP targeting drugs (vincristine and doxorubicin) and non-PGP-targeting drugs (5-fluorouracil and etoposide; ref. 11). In addition, treatment with retinoic acids and other differentiating agents resulted in enhanced expression of the *MDR1* gene product PGP (12). Expression of the *MDR1* gene was also up-regulated by heat shock, arsenate, and serum starvation in cultured human cancer cells (13–16). Consistent with these findings, *MDR1* gene expression was markedly induced by anticancer agents (17); the gene promoter was also activated in response to both anticancer agents and UV light (18, 19). These results show that *MDR1* gene expression is highly susceptible to various environmental stimuli (Table 1) and might therefore be stress responsive (11).

This review focuses on the molecular mechanism of the transcriptional regulation of human *MDR1*/PGP and the role of YB-1 expression in the acquisition of drug resistance.

Transcriptional Regulation of the Human *MDR1* Gene

Many studies have shown the involvement of various *cis*-acting elements in *MDR1* gene expression, suggesting

Received 5/21/04; revised 8/11/04; accepted 8/27/04

Grant support: Ministry of Education, Science, Technology, Sports and Culture of Japan and Ministry of Health and Welfare of Japan.

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Table 1. Transcriptional regulation of the *MDR1* gene in human cell lines

Transcription factor	Inducers	References
NF-Y	None	(40)
	Sodium butylate	(43)
	Trichostatin A	(42)
Sp1	None	(45)
YB-1	UV light	(30, 32)
	Anticancer agents	(31)
Nuclear factor-interleukin-6	Phorbol ester	(52, 78)
EGR1	Phorbol ester	(45, 46)
HSF1	Heat shock	(15, 55)
20-kDa protein	Serum starvation	(16)
Transcription factor 4/β-catenin	None	(56)
Human T-cell lymphotrophic virus-1 Tax	Virus infection	(79)
SXR	Digoxin	(80)
MDR1 promoter-enhancing factor 1/RNA helicase A	None	(59, 60)
Nuclear factor-κB	Daunomycin	(58)
p53	None	(49–51)

pleiotropic mechanisms (10). As shown in Table 1, several transcription factors are expected to play critical roles in the basal expression of the *MDR1* promoter in addition to stimulus-induced activation.

Y-Box Binding Protein 1

Many reports on the factors associated with drug resistance have shown a plausible association of YB-1 with drug resistance both in cultured cancer cells and in numerous clinical human tumor samples.

YB-1 is a member of the cold shock domain (CSD) protein family, which is found in the cytoplasm and nucleus of mammalian cells. It has pleiotropic functions in the regulation of gene transcription and translation, DNA repair, drug resistance, and cellular responses to environmental stimuli (20–22). The structures of YB-1 and two other members of the CSD family, hdbpA (23) and Contrin/hdbpC (24), are presented in Fig. 1A. The *YB-1* gene, which is located on chromosome 1p34 (25, 26), contains eight exons spanning 19 kb of genomic DNA (Fig. 1B). The 1.5-kb mRNA encodes a 43-kDa protein comprising three domains: a variable NH_2-terminal tail domain (A/P domain), a highly conserved nucleic acid binding CSD, and a COOH-terminal tail domain (B/A repeat; refs. 27–29). The A/P domain (amino acids 1-51) seems to be involved in transcriptional regulation, whereas the CSD domain and part of the B/A repeat (amino acids 51-205) function in binding the Y-box (inverted CCAAT box) or double-stranded DNA. Most of the COOH-terminal region of the B/A repeat domain (amino acids 129-324) is thought to bind ssDNA or RNA, and part of this region (amino acids 129-205) is involved in dimerization.

We identified YB-1 as a transcription factor that binds to the inverted CCAAT box of the *MDR1* promoter (30). Decreased expression of YB-1, resulting from the introduction of YB-1 antisense expression constructs into cancer cells, markedly reduced the activation of the *MDR1* gene by DNA-damaging agents (31).

YB-1 is normally present in the cytoplasm, although it is translocated to the nucleus when cells are exposed to anticancer agents, hyperthermia, or UV light irradiation (19, 32, 33). YB-1 is often overexpressed in malignant cells and its expression is regulated by both the proto-oncogene product c-Myc and the tumor suppression gene product p73 (25, 34). The COOH-terminal tail domain seems to play a key role in the localization of YB-1 to either the cytoplasm or the nucleus (32). Studies have shown that cell cycle–specific nuclear translocation is mediated by cooperation of the CSD and COOH-terminal tail domain (35) and that the nuclear translocation of YB-1 requires wild-type p53 (36). The introduction of antisense RNA into human cancer cell lines,[10] and the targeted disruption of one Y-box allele in chicken DT40 cells (37) both inhibited growth. By contrast, the targeted disruption of one allele of the *YB-1* gene in mouse ES-1 cells had no effect on the growth rate (38).

Nuclear Factor Y

The CCAAT box is among the most ubiquitous DNA elements in both forward and reverse orientation. NF-Y is the major transcription factor recognizing the CCAAT box (39). This heteromeric protein is composed of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA binding (Fig. 1A). Mutation and/or deletion of the CCAAT box have been shown to result in a significant loss of *MDR1* promoter activity (40). It has been reported that both the inverted CCAAT box and the GC box are required for activation of the *MDR1* promoter by UV light, and NF-Y, not YB-1, is thought to be the factor regulating the *MDR1* gene (41). However, these findings are not consistent with the results discussed above. The YB-1 protein is abundant and localized in the cytoplasm; however, when the effect of YB-1 overexpression on *MDR1* promoter activity was evaluated in human cancer KB cells, it was unclear whether the nuclear YB-1 content was increased. As YB-1 is known to repress translation, increased levels of cytoplasmic YB-1 might inhibit the translation of luciferase mRNA. Further studies are required to resolve this issue. Treatment with a histone deacetylase inhibitor (trichostatin) induced a marked increase in the amount of *MDR1* mRNA, although this drug-induced increase was inhibited in dominant-negative NF-Y mutants (42). NF-Y therefore seems to regulate *MDR1* gene expression through an interaction with p300/CBP-associated factor, which shows histone acetylation activity. NF-Y might also be responsible for the sodium butyrate–induced *MDR1* gene up-regulation in colon cancer cells (43). This transcription factor therefore plays a pivotal role in *MDR1* gene expression. Recently, the antitumor agent HMN-176, which interacts with NF-YB, has been shown to inhibit *MDR1* gene expression and to restore chemosensitivity to MDR cells (44).

[10] K. Kohno and M. Kuwano, unpublished data.

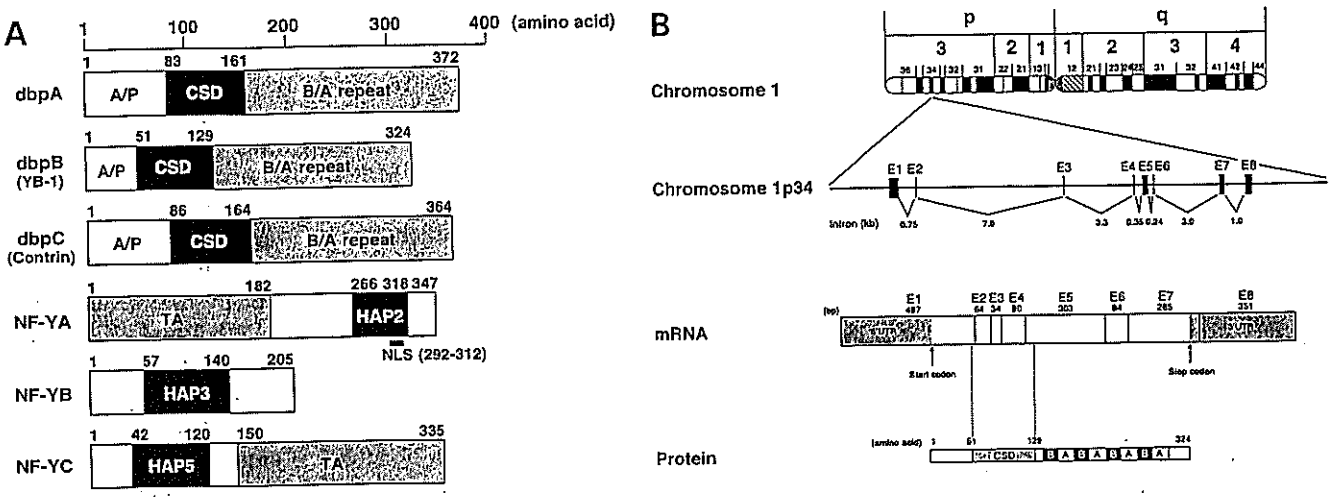

Figure 1. A, protein structure and functional domains of hdbpB/YB-1, hdbpA, hdbpC, NF-YA, NF-YB, and NF-YC. *A/P,* alanine and proline domain, residues 1-82, 1-50, and 1-85 in hdbpA, hdbpB/YB-1, and Contrin/hdbpC, respectively. *CSD,* residues 83-161, 51-129, and 86-164. *B/A repeat,* basic and acidic amino acid, residues 162-372, 130-324, and 165-364. The CSD domains of the three genes are highly homologous. Of the three subunits of NF-Y, NF-YB and NF-YC contain histone folding motifs homologous to the yeast transcription factors *HAP3* and *HAP5*, respectively. NF-YA contains a domain homologous to *HAP2*, which interacts with NF-YB and NF-YC, and the heterotrimer of NF-Y binds to DNA. Both NF-YA and NF-YC contain glutamine-rich domains and activate transcription. B, general structure of the genomic DNA, mRNA, and protein product of *YB-1*. The gene is mapped at chromosome 1p34 and has eight exons (*E1, E2, E3, E4, E5, E6, E7,* and *E8*). The YB-1 protein consists of 324 amino acids. *B,* basic amino acid clusters; *A,* acidic amino acid clusters.

Sp1 and Early Growth Response Element 1

The introduction of mutations in the GC-rich region −59 to −45 (G region) of the *MDR1* promoter markedly decreased its activity as a result of the transcription factor Sp1 (40, 45). Sp1 was first cloned and identified as a transcription factor specifically bound to the GC box of the SV40 promoter. A GC box is found in the promoter region of many eukaryotic genes. The Sp1 family is involved in various cellular functions including proliferation, apoptosis, differentiation, and neoplastic changes. As the early growth response element 1 (EGR1) binding motif partially overlaps with the Sp1 binding sites, it is conceivable that they mutually influence *MDR1* gene expression in a competitive manner (45). Treatment with phorbol ester induced the expression of both *EGR1* and *MDR1* genes in human leukemia cells (46). However, the expression of *EGR1* alone did not enhance *MDR1* promoter activity. Coexpression of the oncosuppressor gene *WT1* resulted in the inhibition of *MDR1* promoter activation by EGR1 or phorbol ester (47). Therefore, the direct binding of WT1 to the GC box might compete with Sp1 to down-regulate the *MDR1* gene. These findings suggest that interactions between EGR1 and WT1 might play a key role in *MDR1* promoter activation.

p53

Mutant p53 has been shown to enhance *MDR1* promoter activity in mouse cells; this was reversed by wild-type p53 (14, 48). By contrast, stimulation of the *MDR1* promoter by wild-type, but not mutant, p53 was shown in several human p53-null cancer cell lines. The *MDR1* promoter region −39 to +53 is responsible for this p53-mediated activation (49), whereas the region −189 to +133 is thought to be responsible for negative regulation by wild-type p53 (50). In addition, p53 has been reported recently to bind directly to a novel binding element (−72 to −40) within the *MDR1* core promoter and to repress its promoter activity (51).

Nuclear Factor-Interleukin-6

The treatment of human monocytic cells with phorbol ester enhanced *MDR1* promoter activity through interaction with nuclear factor-interleukin-6, which is a CCAAT/enhancer binding protein family member. This study also revealed that the mitogen-activated protein kinase pathway activates nuclear factor-interleukin-6 (52). In addition, CCAAT/enhancer binding protein β has been shown recently to transactivate the *MDR1* promoter by interaction with the Y-box (53).

Heat Shock Factor

MDR1 promoter activation in response to arsenate or heat shock seems to be mediated through a heat shock element in the −178 to −165 region. An additional region at −136 to −76 has also been proposed as a critical heat shock element for the heat shock response (15, 54), although no direct binding of heat shock factor to this region has been shown. Recently, Vilaboa et al. (55) reported that infection with adenovirus carrying heat shock transcription factor 1 cDNA increased the levels of *MDR1* mRNA and PGP.

Transcription Factor 4/β-Catenin

Transcriptional profiles produced using cDNA microarrays in human colon cancer cell lines identified the *MDR1* gene as the target of transcription factor 4/β-catenin. Seven transcription factor 4/β-catenin binding sites were in the promoter region between −2,030 and +31 (56).

Nuclear Factor-κB

The hepatocarcinogen 2-acetylaminofluorene was shown to activate the *MDR1* gene in human hepatoma cells and the induction of *MDR1* by 2-acetylaminofluorene was mediated by a nuclear factor-κB binding site located around −6 kb (57). Another group showed that the inhibition of nuclear factor-κB reduced levels of *MDR1* mRNA and PGP expression and that nuclear factor-κB transactivated the *MDR1* promoter in human colon cancer HCT15 cells (58). This study identified a nuclear factor-κB binding site in the first intron.

MDR1 Promoter-Enhancing Factor 1/RNA Helicase A

MDR1 promoter-enhancing factor 1 has been shown to bind to the CCAAT sequence causing up-regulation of the *MDR1* gene (59). RNA helicase A has also been reported to bind to the CCAAT box as a member of the *MDR1* promoter-enhancing factor 1 complex (60). Overexpression of RNA helicase A enhanced the expression of both the *MDR1* promoter-reporter construct and endogenous PGP.

Clinical Implications of PGP Expression and Nuclear Translocation of YB-1

PGP triggers resistance to a wide range of anticancer agents including *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxols (7). In addition, YB-1 plays a role in limiting the drug sensitivity of cancer cells by increasing the expression of PGP and other proteins. Immunohistochemical studies of YB-1 expression in the nuclei of untreated primary breast cancers showed an almost complete association between nuclear YB-1 and PGP expression in 9 of 27 cases (Table 2; ref. 61). Studies of clinical specimens have also shown an association between YB-1 and PGP in osteosarcoma (62), synovial sarcoma (63), breast cancer (64, 65), ovarian cancer (66–68), and prostate cancer (Table 2; ref. 69). Figure 2 shows examples of the presence and absence of YB-1 and PGP in clinical samples of osteosarcoma and synovial sarcoma based on the results of immunohistochemical analyses with anti-YB-1 and anti-PGP antibodies.

Table 2. The association of nuclear expression of YB-1 with PGP-mediated and/or non-PGP-mediated drug resistance in human malignancies

Tumor type	Malignant characteristics	References
Ovarian cancer	PGP* ↑	(66)
	PGP* ↑	(67)
	Cisplatin resistance	(68)
Breast cancer	PGP ↑	(61)
	PGP* ↑	(64)
	Drug resistance	(65)
Osteosarcoma	PGP ↑	(62)
Synovial sarcoma	PGP* ↑	(63)
Prostate cancer	PGP* ↑	(69)

*These studies also reported a significant correlation between nuclear YB-1 expression and disease progression or prognosis.

There was a significant correlation between the nuclear expression of YB-1 and the presence of PGP in 69 cases of osteosarcomas (62). A recent study confirmed that YB-1 expression was specifically associated with the overexpression of PGP rather than with three other ATP binding cassette transporters: MRP1, MRP2, and MRP3 (63). By contrast, no association was observed between YB-1 and PGP expression in colon cancers (70). It remains unclear whether YB-1 is directly involved in the transcriptional regulation of PGP in human malignancies. Nevertheless, measurements of the expression of YB-1 and PGP could suggest treatment modalities for individual cancer patients. Recently, we showed that coexpression of YB-1 and PGP correlated with poor prognosis in epithelial ovarian cancer (67). The expression of *MDR1* is augmented in cancerous areas in breast cancer and other tumors, resulting in drug resistance. Furthermore, the presence of YB-1 in the nuclei of cancer cells is closely associated with the clinical outcome. YB-1 could therefore be a useful indicator of malignancy as well as a promising target for cancer therapy. We recommend the use of non-PGP-targeting drugs against malignant tumors with nuclear YB-1 expression.

Clinical Implications of Nuclear Localization of YB-1: Drug Resistance to non-PGP-Targeting Drugs

As described above, YB-1 is translocated to the nucleus in response to various environmental stresses including UV light, anticancer agents, heat, and infection in cultures of cancer cells (21). YB-1 was shown to be overexpressed in cisplatin-resistant cell lines, and antisense YB-1 RNA triggered the augmentation of sensitivity to cisplatin, mitomycin C, UV light, and hydrogen peroxide (30, 38). YB-1 associates with p53 (71) and proliferating cell nuclear antigen (72), both of which modulate DNA repair, cell cycle, transcription, and drug sensitivity. Moreover, wild-type p53 is required for the nuclear translocation of YB-1, which in turn inhibits p53-induced cell death (36). However, it remains unclear how reduced YB-1 expression increases resistance to non-PGP-targeting DNA-damaging agents such as cisplatin and mitomycin C. Potential mechanisms might include a reduction in the YB-1 interaction with proliferating cell nuclear antigen, which is necessary for nucleotide excision repair, or in the interaction with p53. However, pleiotropic drug resistance to DNA-interacting drugs (e.g., aphidicolin, hydroxyurea, cytarabine, etoposide, doxorubicin, and mafosfamide) is associated with the increased expression of YB-1 and 19 other genes that are involved in DNA replication, repair, and stress responses (73).

Nuclear expression of YB-1 was reported to be a prognostic factor in ovarian serous adenocarcinoma (66). It was also associated with cisplatin resistance in ovarian cancer cell lines, and expression levels were increased at some sites of ovarian cancer recurrence (68). This pattern was seen in 7 of 21 serous adenocarcinomas, 2 of 7 clear cell

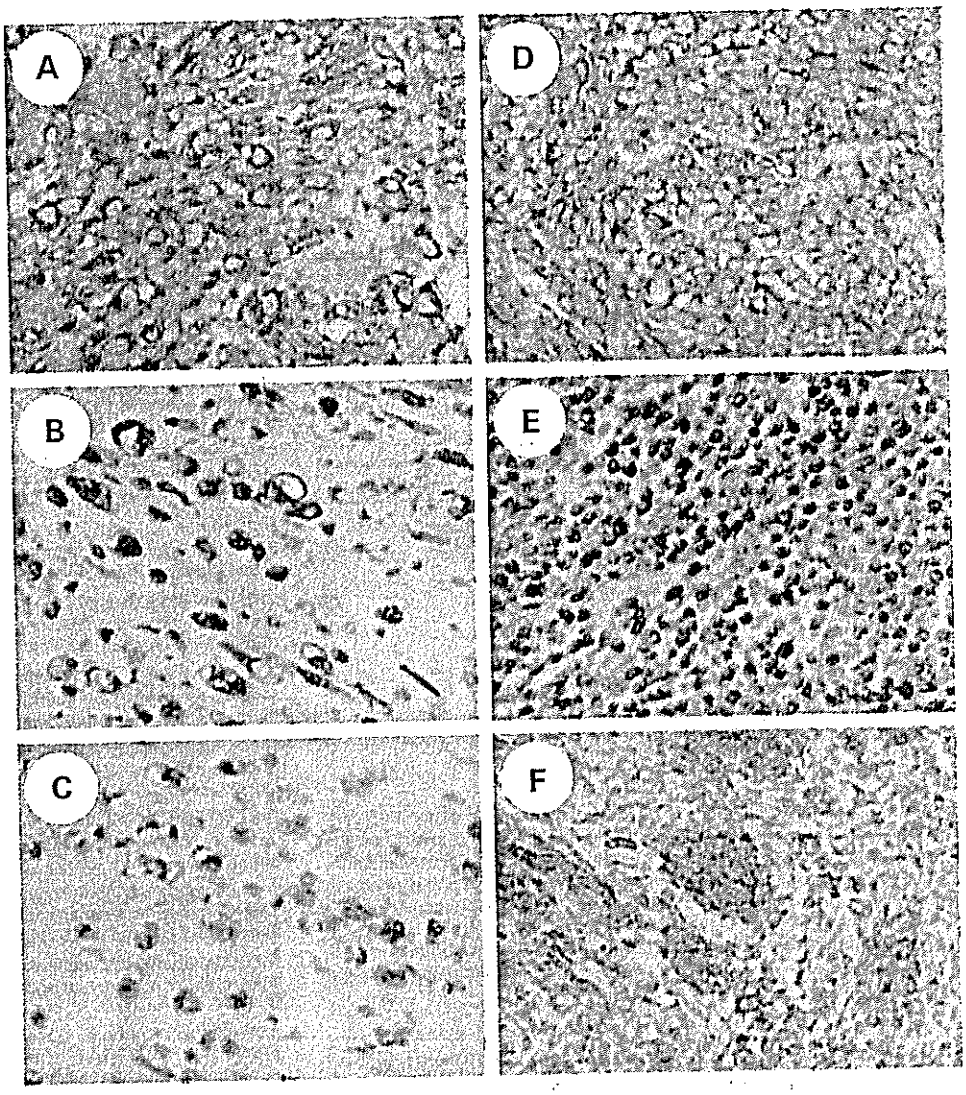

Figure 2. Immunohistochemical detection of nuclear and cytoplasmic YB-1 in osteosarcoma and synovial sarcoma. Antibodies were used against YB-1 (A, B, D, and E) or PGP (C and F). Osteosarcoma is shown with cytoplasmic YB-1 expression (A), nuclear YB-1 expression (B), and PGP expression (C). Synovial sarcoma is shown with cytoplasmic YB-1 expression (D), nuclear YB-1 expression (E), and PGP expression (F). The patient in D showed no evidence of disease 131 months after surgery. The patient in F died of lung metastasis 8 months after the initial surgery.

adenocarcinomas, and 1 of 4 mucinous adenocarcinomas (Table 2). There was also a positive correlation between the nuclear expression of YB-1 and poor prognosis in synovial sarcoma (63).

Analysis of the clinical relevance of YB-1 expression in the cytoplasm or nucleus in 83 cases of breast cancer, after a median follow-up of 61 months, revealed that the 5-year relapse rate was 66% in patients with high YB-1 expression who received postoperative chemotherapy (65). By contrast, none of the patients with low YB-1 expression experienced relapse. Taken together, these findings indicate that the overexpression and nuclear expression of YB-1 have a predictive value in some human malignancies, both with and without postoperative chemotherapy.

An investigation of 588 genes associated with mouse lung tumor progression revealed that 19 were differentially expressed between lung adenoma and adenocarcinoma; YB-1 was one of these candidate lung tumor progression genes (74). Overexpression of YB-1 was observed in >90% of anaplastic thyroid carcinomas, whereas it was absent in normal follicles and other pathologic tumor types. These findings suggested the involvement of YB-1 in the anaplastic transformation of thyroid carcinoma (75). YB-1 expression induced a strong cellular resistance to malignant transformation through the phosphatidylinositol 3-kinase pathway possibly through the inhibition of protein synthesis that is required for the phosphatidylinositol 3-kinase– or Akt-induced oncogenic transformation (76).

Conclusion

The ancestral protein YB-1 modulates cell growth, apoptosis, drug resistance, DNA repair, transcription, and translation as a pleiotropic regulator. YB-1 overexpression or nuclear YB-1 expression might play a key role not only in the acquirement of PGP-mediated drug resistance but also in sensitivity to non-PGP-targeting chemotherapeutic agents. YB-1 in the nucleus modulates drug resistance to PGP-targeting and non-PGP-targeting drugs in cancer cells

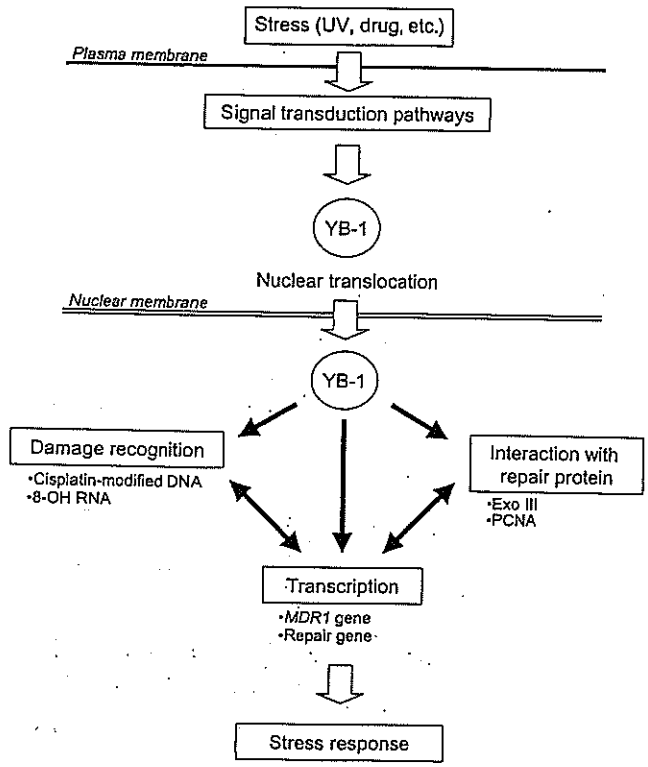

Figure 3. Schematic summary of MDR mediated by PGP or non-PGP. YB-1 is normally present in the cytoplasm but is translocated to the nucleus by treatment with anticancer agents, hyperthermia, or UV light irradiation. YB-1 in the nucleus functions as a transcription factor, which can bind to the Y-box and transactivate promoters, such as the *MDR1* gene or repair genes. By contrast, YB-1 can bind directly to cisplatin-modified DNA and interact with repair proteins including NTH1 (*Exo III*) and proliferating cell nuclear antigen (*PCNA*). These functions might be advantageous for the acquisition of drug resistance.

that are exposed to anticancer and other cytotoxic DNA-damaging agents (Fig. 3). In one response pathway to environmental stimuli, YB-1 is translocated to the nucleus and up-regulates *MDR1* gene expression through binding to the Y-box on the promoter. Alternatively, YB-1 might operate its DNA repair pathway through interactions with p53 (71), proliferating cell nuclear antigen (72), and other molecules (77) when DNA is damaged (Fig. 3). Further research is needed to fully understand the role of YB-1 in cancer and drug resistance.

Acknowledgments

We thank our colleagues for invaluable help in completing the studies presented in this article.

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特集／トランスポーター

7．多剤耐性トランスポーター：抗がん剤のMDR

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1．はじめに

薬剤を感染症やがん患者の治療のために投与を続けると，しばしば薬剤効果の低下が観察される．抗生物質に対する耐性病原細菌の出現ならびに抗がん剤に対する耐性がんの出現は，化学療法が始まって以来，長い治療戦略の歴史とともに簡単に解決できない大きな難問として現在まで続いている．病原細菌とがん細胞の耐性について多くの共通するメカニズムが存在すると同時に，各々特徴的なメカニズムも存在している．とくに本稿では，構造や作用点の異なる多くの抗がん剤に同時に耐性を示すがんの多剤耐性[1]について，P糖蛋白質/MDR1遺伝子を中心に，さらにMRP遺伝子（本特集の野崎らの項 p. 81 参照）などの関与について一部述べる．

P糖蛋白質やその他のABCトランスポーター（MRP1やBCRPなどを含む）の発現上昇は，抗がん剤やその他薬剤に対する耐性で選択した耐性体細胞株でしばしば観察される．Victor Ling博士はvincristine耐性のハムスター細胞株を，秋山伸一博士はcolchicine耐性のヒトがん細胞を各々単離樹立した．さまざまな薬剤の排出を亢進させる多剤耐性の獲得に寄与するABCトランスポーターMDR1遺伝子が，Victor Ling博士やIra Pastan博士と植田和光博士らの研究グループによって単離された．我々の研究室で単離した2種類のヒトがん細胞由来の多剤耐性細胞株KB/VJ300とKB/VM4は，各々選択に用いた薬剤に耐性を示すだけでなく，他薬剤に対しても交差耐性を示している[2,3]（Table 1）．KB/VJ300ではMDR1遺伝子の発現上昇が，KB/VM4ではMRP1遺伝子発現上昇が各々観察され，両ABCトランスポーター遺伝子が多剤耐性の獲得に寄与していることが示されている．

Table 1　ヒトがん細胞より樹立したP糖蛋白質（KB/VJ300）とMRP1（KB/VM4）発現の多剤耐性細胞株の抗がん剤に対する耐性パターン[a]

抗がん剤	KB/VJ300	KB/VM4
doxorubicin	10	14
vincristine	400[b]	2
actinomycin-D	100	2
daunorubicin	20	9
etoposide（VP16）	5	150
teniposide（VM26）	5	110[b]
camptothecine	4	1
cisplatin	1	0.5

a）：細胞集落形成能における10%生存率から親株KB3-1を1.0としたときの相対的薬剤濃度を示す．
b）：KB/VJ300とKB/VM4は各々vincristineとteniposideで選択した細胞株でP糖蛋白質/MDR1とMRP1が各々優位に発現している．

現在，50個近いABCトランスポーターが単離され，その中には研究が進み抗がん剤の耐性や細胞外排出に関与するトランスポーターが同定されている[4,5]．薬剤耐性で選択された多剤耐性細胞やcDNA導入強制発現株，また動物のノックアウトマウスなどの実験結果から明らかにされてきたP糖蛋白質をはじめとする4つのトランスポーターと関連する抗がん剤をTable 2に示している[6,7]．その中でも，P糖蛋

Key words：multidrug resistance, P-glycoprotein, MDR1, MRP1, ABC transporter
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Table 2 P糖蛋白質，MRP2，MRP3およびBCRPが認識し耐性と関連する抗がん剤

P糖蛋白質	MRP2	MRP3	BCRP
doxorubicin	doxorubicin	etoposide	vincristine
daunorubicin	vincristine	teniposide	camptothecine
etoposide	camptothecine	methotrexate	mitoxantrone
teniposide	cisplatin		
vincristine	methotrexate		
paclitaxel			
actinomycin-D			

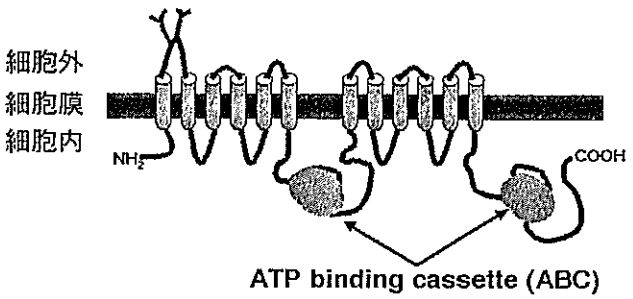

Fig. 1 P糖蛋白質の構造

白質とMRP1は遺伝子が単離されたのが古いこともあって，抗がん剤の多剤耐性への関与に関する研究が進んでいる．

2．MDR1/P糖蛋白質と多剤耐性

P糖蛋白質は，嚢胞性線維症（cystic fibrosis）の原因遺伝子CFTRと並んで，最も研究の進んでいるヒトABCトランスポーターである(Fig. 1)．Alvarezら[8]は，60種類のヒトがん細胞株で抗がん剤の感受性をスクリーニングした結果，感受性に関与する鍵を握る最も重要な分子標的はP糖蛋白質であったと報告している（Table 1, 2参照）．P糖蛋白質は，肝，膵，腎，副腎，子宮(妊娠時)，精巣，胎盤，卵巣，腸，骨格筋などの正常器官や組織で発現しており，ステロイドをはじめ多くの生理物質の輸送や排泄にかかわっている（Table 3）．さらに，Peter Borst博士らのグループによるノックアウトマウスの実験からも示されたように，脳血液関門など生体内外の毒物などに対しても，生体防御の重要な働きを担っている[9]．

MDR1遺伝子の発現の多剤耐性の獲得への寄与について，培養系のがん細胞などで得られた情報のほかに個体レベルで証明したのはIra Pastan博士らの研究グループの発表である[10]．彼らはMDR1遺伝子を，骨髄系細胞に選択的に発現する耐性トランスジェニックモデルマウスを作製し，鶴尾隆博士らによって開発

Table 3 ヒト正常組織とがんにおけるP糖蛋白質とMRP1の発現

	P糖蛋白質	MRP1
正常組織	副腎，子宮(妊娠時)，腎，脳，肝，大腸	胎盤，心，精巣，単球，肺，骨格筋
がん	白血病，大腸がん，リンパ腫，腎がん，乳がん，肺がん，膵がん，卵巣がん	神経芽腫，甲状腺がん，リンパ腫，食道がん，白血病，腎がん，肺がん，乳がん

されていたP糖蛋白質に特異的なモノクローナル抗体と緑膿菌毒素のキメラ蛋白の投与によって，白血球数の減少がみられることを報告した．さらにこのマウスに，daunorubicin単独投与では白血球数の減少はみられないが，P糖蛋白質の機能を抑えるverapamilを併用すると著明な白血球数の減少が観察された．以上の実験から，MDR1遺伝子が確かに個体レベルでも，“耐性獲得”に関与していることが明らかにされたわけである．

ヒトMDR1遺伝子は染色体7q21.1上に位置しており，Fig. 2にゲノム構造とMDR1遺伝子の5′-制御領域について示している[11]．ヒトMDR1やMDR3を含むゲノム500キロベース(kb)を酵母染色体に連結させて酵母人工染色体(YAC)を作製し，マウス細胞に導入し，vincristine存在下に多段階に濃度を上昇させて，数倍から数百倍の耐性度の異なるマウス耐性細胞株を単離した．その結果，マウス多剤耐性遺伝子の発現上昇は全くみられないときに，YAC上のヒトMDR1遺伝子領域が特異的に遺伝子増幅と発現上昇がみられることが観察された．ヒト染色体7q21.1上のMDR1ゲノム領域が，“多剤耐性”の獲得に必須なゲノム情報をもっていることが明らかになった．

MDR1遺伝子は，多くの悪性腫瘍で発現していることが知られている（Table 3）．MDR1遺伝子の発現上昇の機序に関して，培養系で耐性選択されたMDR1遺伝子の過剰発現分子機序の研究が進められた．その結果，MDR1遺伝子の遺伝子増幅，5′-制御領域の遺伝子再編成や転写発現亢進などによる制御機序について発表された．しかし，それらの機序のうち，がん患者におけるMDR1遺伝子発現の上昇に直接関与するものは，それぞれの分子的背景であるのかを明らかにすることはがんの多剤耐性を把握するうえで極めて大切なことである．とくに転写因子YB-1（Y-box結合蛋白-1）やプロモーター上のCpGサイトのメチル化の有無がMDR1遺伝子と相関する臨床研

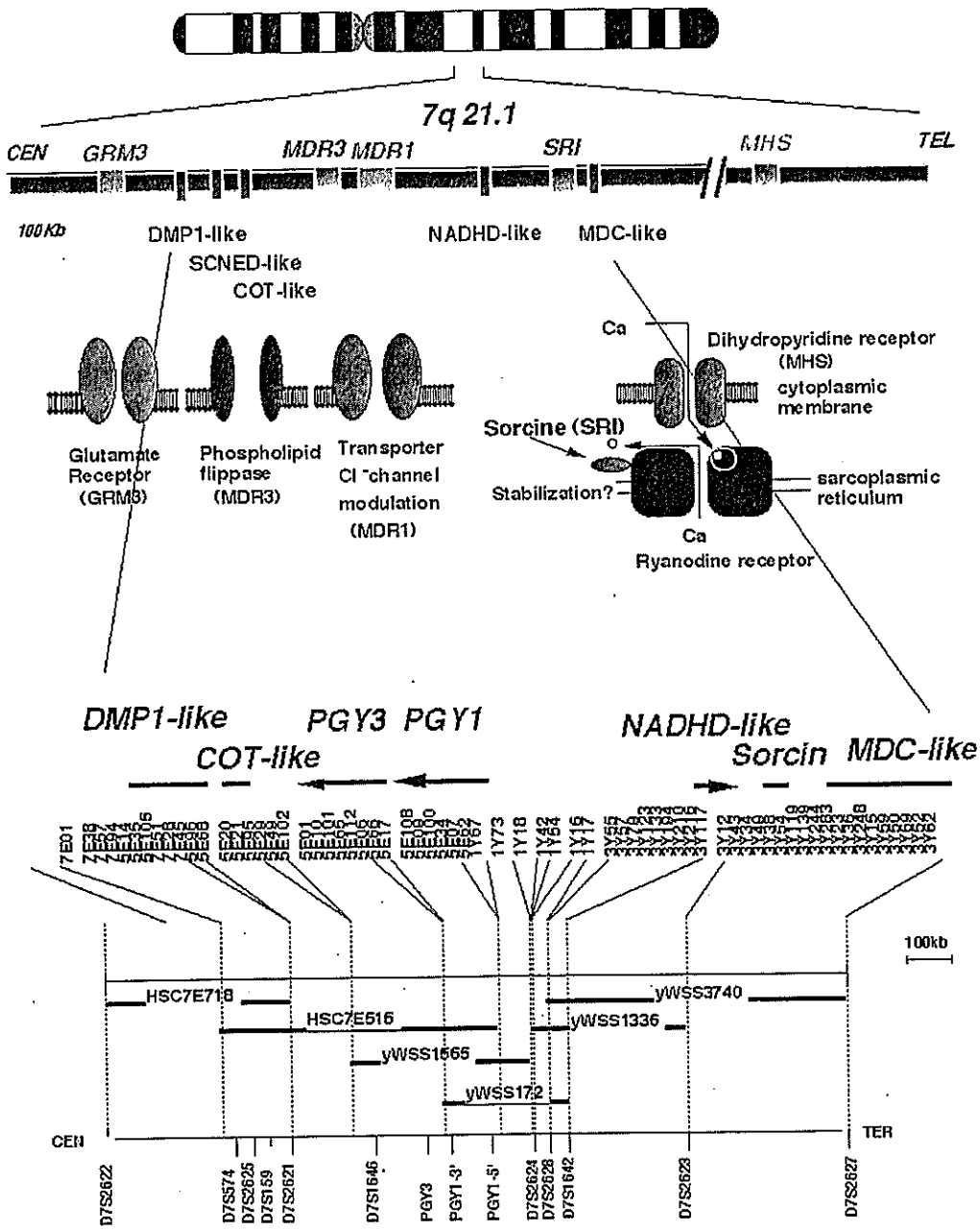

Fig. 2 ヒト7番染色体 q21.1 におけるゲノム再編成

がん化や抗がん剤耐性に伴う MDR1 遺伝子周辺のゲノム再編成や遺伝子増幅や遺伝子発現などを把握するために，MDR1 遺伝子領域の 1.5 Mb の構造を明らかにした．YAC クローンからエクソン・トラップ法によってエクソンの単離を試みている．MDR1（PGY1）ともう1つの ABC トランスポーターMDR3（PGY3）は約 30 kb 離れて存在している．

究について，我々の研究成果を中心に言及する．

我々は，MDR1 遺伝子の発現が抗がん剤や DNA 障害ストレスによって誘導されることを培養系がん細胞で最初に報告した[12]．その分子機序を理解するために研究を進めた結果，MDR1 遺伝子プロモーター上で存在する CCAAT 逆向き配列（Y-box と呼ばれる）に結合する蛋白として YB-1 を単離した．YB-1 は熱ショック蛋白ドメインを大腸菌からヒトまで進化的に保存された原始蛋白であり，細胞質や核内に局在して転写，翻訳，DNA 修復・複製などの多岐にわたる働きを示すことが明らかにされはじめている[6,13,14]．とくに紫外線照射や cisplatin 処理などによる YB-1 の核内移行が MDR1 発現を誘導したり，また YB-1 の核内発現レベルの減少が外界ストレスによる MDR1 発現誘導を減少させるなどの基礎的知見が蓄積してきた．