group compared with those in controls, and MC treatment did not influence the values. Blood homocysteine concentrations were significantly reduced in diabetic group compared with that in normal control group, and MC treatment did not significantly influence the values although the average value was less in the treated group than that in untreated group.

Nerve sorbitol was increased more than 12-fold in diabetic group compared with normal controls, and MC treatment slightly but significantly suppressed this elevation ($P < 0{\cdot}05$) (Table 1). Nerve fructose was increased ninefold in diabetic group compared with controls. MC treatment slightly decreased the average value, but it was not significant.

Nerve conduction velocity

Diabetic group showed 26% reduction in MNCV and 30% reduction in SNCV compared with those in normal control rats, respectively ($P < 0{\cdot}01$ for both)(Fig. 1). MC treatment improved both MNCV by 15% ($P < 0{\cdot}01$ vs. untreated diabetic rats) and SNCV by 22% ($P < 0{\cdot}01$ vs. untreated diabetic rats).

PKC alterations

Endoneurial nerve homogenates were separated into membrane and cytosolic fractions. PKC activity of the membrane fraction was significantly reduced to 75% level in diabetic rats compared with the level of control rats ($P < 0{\cdot}01$), while MC treatment showed a significant recovery of the activity to supranormal levels ($P < 0{\cdot}01$ vs. untreated diabetic rats) (Fig. 2a). On the other hand, PKC activity of the cytosolic fraction was not significantly altered in diabetic rats.

Western blot analysis disclosed that PKCα was reduced in the membrane fraction from diabetic rats, while PKCα in the cytosolic reaction tended to increase (Fig. 2b). MC treatment corrected these changes in both membrane and cytosolic fractions. There was an elevated expression of PKCβII in the membrane fraction in diabetic rats. The cytosolic fractions of PKCβI appeared to be reduced. MC treatment corrected these changes. Comparison of the average values obtained from densitometric analysis among the groups confirmed these changes, although the changes in PKCβI were not significant (Fig. 2c).

Immunohistochemistry

Immunohistochemically, PKCα and PKCβ (both I and II) were differentially expressed in the nerve. PKCα was expressed in the Schwann cells, whereas PKCβI and II located in the membrane of axoplasm, macrophages and the wall of endoneurial microvessels (Fig. 3). There was a trend towards less expressions of PKCα and more intensified expressions of PKCβII in diabetic rats compared with those in normal control rats.

8-Hydroxydeoxyguanosine-positive cells were marked in diabetic rats compared with normal control rats, and this expression was suppressed in MC-treated diabetic rats (Fig. 4a). In normal control rats, there were no or only a few endoneurial Iba-1-positive macrophages (Fig. 4b). In contrast, diabetic rats showed an abundance of Iba-1-positive cells. MC treatment slightly suppressed the migration of macrophages, but it was not significant. Quantitative analysis confirmed the earlier findings (Fig. 4c).

Discussion

This study confirmed a significant NCV delay and decreased PKC activity in peripheral nerve in STZ-induced diabetic rats. The reduction in PKC activity was associated with reduced expression of membranous PKCα [21,22]. In contrast, membranous PKCβII expression was increased in diabetic rats, but this change was not reflected by total PKC activity. MC treatment effectively corrected the PKC activity and expression of PKCα, together with improved NCV. It is noteworthy that the localization of PKC isoform was quite distinct between PKCα of Schwann cells and PKCβ of axoplasm.

Protein kinase C is crucial to mediate cellular signalling and to stimulate cell activity, protein synthesis and cell movement [23,24]. PKC consists of many isoforms; α, βI, βII, γ, δ and others. Increased PKC activity owing to augmented PKCβ expressions has consistently been demonstrated in cardiovascular system in diabetic subjects [25,26], and as such clinical trial of PKCβ inhibitor for the treatment of diabetic neuropathy was conducted [18,19]. It was not successful, however, in part because of the fact that the role of PKC in neuropathic processes is yet to be established. In fact, PKC activity in diabetic nerves is differently reported in previous studies as elevated [27,28], reduced [20,21] or not altered [29]. Our present results may explain the reason for such contradictory results by differential expression of PKC isoform. First, we confirmed our previous findings of decreased PKC activity in the endoneurial fraction of STZ-induced diabetic nerve [21,22,30], while epineurial vascular system showed elevated PKC activity with PKCβ overexpression [30]. Localization of PKCα in Schwann cells found in this study was consistent with the data that cultured Schwann cells exposed to high glucose showed decreased PKC activity with reduced expression of PKCα [31]. On the other hand, increased phosphorylation of PKCβ in dorsal root ganglion cells that was found in STZ-diabetic rats with elevated pain sensation [27] may also be in agreement with our results of the strong immunoreactions of PKCβ in axoplasmic membrane in diabetic rats. It is therefore likely that although total nerve PKC activity may be suppressed, the beneficial effects of PKCβ inhibitor previously reported [29] may be ascribed to the amelioration of nerve blood flow as well as direct action of PKCβ in the soma of ganglion cells and axons shown in this study.

In previous studies, the altered PKC activity was closely correlated with increased polyol pathway in diabetic nerve.

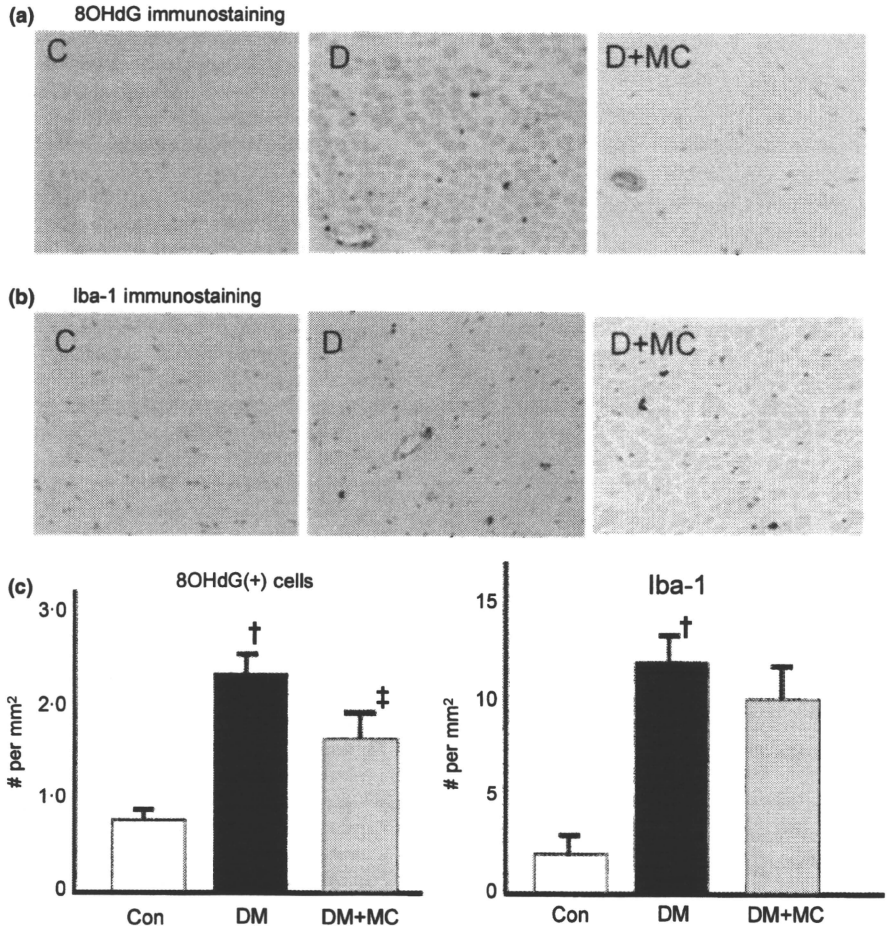

Figure 4 Expression of oxidative stress–induced DNA damage and macrophage migration in the peripheral nerve in experimental animals. (a) As revealed by immunohistochemistry, diabetic rats showed increased number of 8-hydroxydeoxyguanosine (8OHdG)-positive cells in the nerve, and this increase was significantly suppressed in methylcobalamin-treated diabetic rats. (b) In the peripheral nerve, there were an increased number of macrophages positive with Iba-1 immunoreactions in diabetic rats, and there was no significant influence of methylcobalamin treatment on the macrophage migration in untreated diabetic rats. (c) Quantitative estimation confirmed the findings. C, normal control rats; D, untreated diabetic rats; D+MC, methylcobalamin-treated diabetic rats. Bar stands for SE. †$P < 0{\cdot}01$ vs. C, ‡$P < 0{\cdot}01$ vs. D.

In this study, sorbitol accumulation was only partially inhibited by MC treatment, whereas 8OHdG-positive cells were significantly reduced. It was shown that sorbitol accumulates in tissues not only under hyperglycaemia but also ischaemic/hypoxic milieu [32,33]. In previous studies, sorbitol levels in the sciatic nerve were not influenced by MC despite the significant improvement in NCV in STZ-diabetic rats [16]. It is therefore likely that the recovery of PKC activity in MC-treated animals may not be directly dependent on inhibition of polyol pathway but rather relate to the changes in oxidative stress–induced damage. In this setting, antioxidant effects of MC may be mediated by the synthesis of cysteine donated from transmethylation of methionine [34,35]. Alternatively, activation of metabolic signals as well as protein synthesis by stimulation of nucleic acid metabolism elicited by MC may also have contributed to the improvement in PKC activity [36,37]. The consequence of recovered PKC alterations may directly or indirectly prevent the distal axonal fibre loss in the skin and enhance nerve fibre regeneration. Indeed, recent experimental studies demonstrated increased nerve fibre regeneration after crush injury of the sciatic nerve by MC treatment through the activation of cellular signalling of Akt and MAP kinase [38]. Upregulation of growth factor expressions like IGF-1 in the nerve by MC treatment also resulted in amelioration of neuropathic changes in STZ-diabetic rats [39].

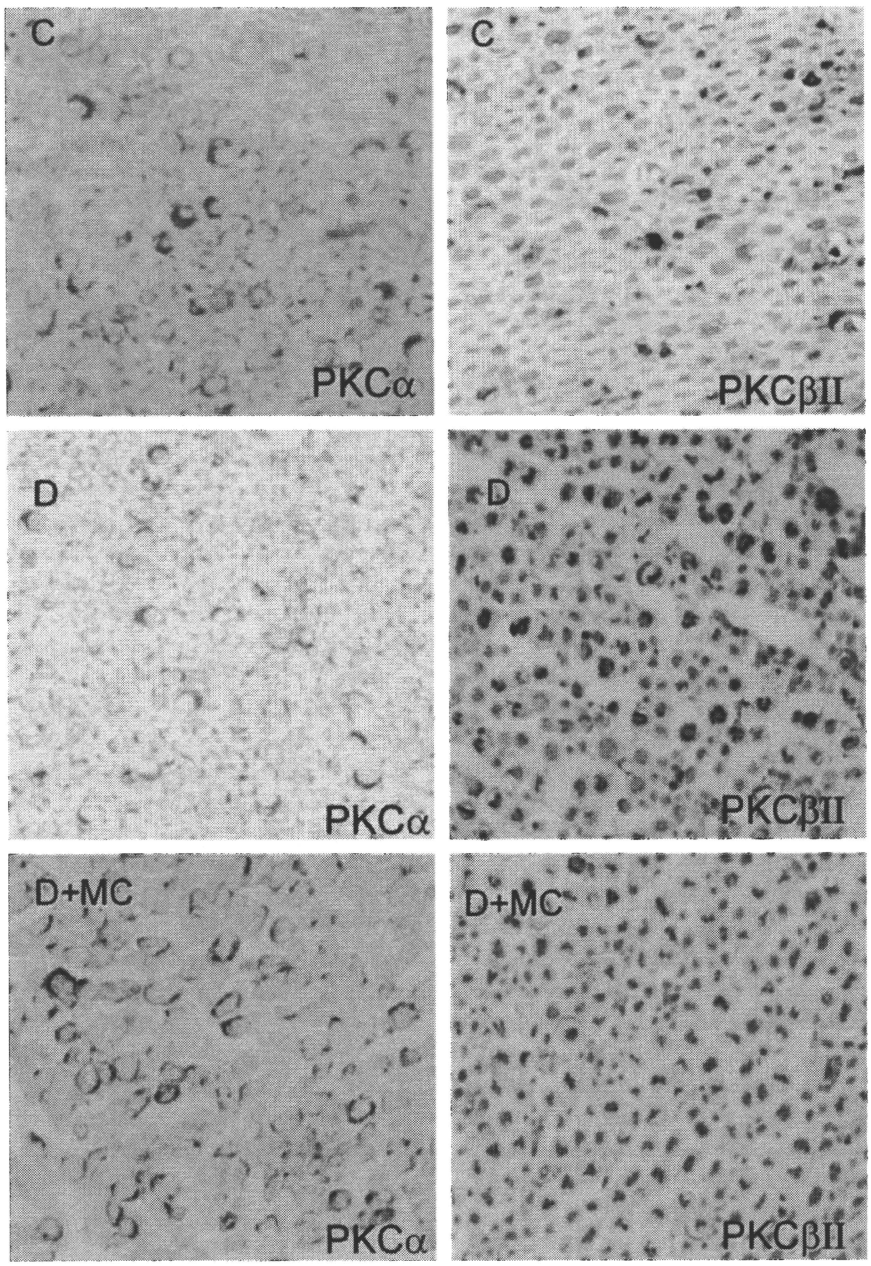

Figure 3 Topography of protein kinase C (PKC) isoform expressions as revealed by immunohistochemistry. PKCα isoform was diffusely localized in the Schwann cell cytoplasm in the peripheral nerve. There was no significant alteration in diabetic nerve, and methylcobalamin treatment did not influence the reactions. On the other hand, PKCβII isoform was localized in the axoplasm of nerve fibres. The membranous reaction of PKCβII isoform was intensified in the axoplasm in diabetic rats, and the changes were less marked in methylcobalamin-treated diabetic rats. C, normal control rats; D, untreated diabetic rats; D+MC, methylcobalamin-treated diabetic rats.

In transgenic mice overexpressing human aldose reductase (AR), PKC activity was severely suppressed, and this change was corrected by AR inhibitor [20,30]. In contrast, pioglitazone improved PKC activity without affecting polyol levels [21]. Instead, it corrected the oxidative stress–induced damage and macrophage migration in the endoneurium [21].

Homocysteine levels in STZ-induced diabetic rats are known to be low in contrast to the elevation in human type 2 diabetic subjects [7,8,10,40]. The reason why homocysteine values are low in STZ-diabetic rats is not clear. Nevertheless, the values were further reduced in MC-treated animals. Hence, it is likely that there is a dynamic regulatory system between homocysteine and methionine by MC treatment, although the processes may be different between nerve and blood.

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Contributions of authors

Mizukami H conducted the experiment and summarized the data (40% contribution), Ogasawara S helped all the experiment with animal care and conducted biochemical analysis (30% contribution), Yamagishi SI measured nerve function and PKC (10% contribution), Takahashi K conducted immunohistochemistry and helped animal care (10% contribution), and Yagihashi S designed the experiment and wrote the manuscript (10% contribution).

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