Prednisolone, Platelet-Activating Factor Receptor Antagonist, or Superoxide Dismutase Reduced Leukocyte Entrapment Induced by Interferon Alpha in Retinal Microcirculation

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**Purpose.** Interferon (IFN) alpha has been suggested as a possible treatment for choroidal neovascularization. However, the pathogenesis of retinal complications after IFN therapy still is unknown. Previously, we have shown that IFN alpha induced leukocyte entrapment in retinal microcirculation. The current study was designed to determine if leukocyte entrapment can be reduced by the agents that modulate leukocyte-endothelial adherence.

**Methods.** Interferon alpha was administered intravenously in rats. Simultaneously, prednisolone (PSL), platelet-activating factor receptor antagonist (CV-6209), or superoxide dismutase (SOD) was given to the rats. Leukocyte dynamics were observed with acridine orange (AO) digital fluorography, which uses a nuclear fluorescent dye of AO and scanning laser ophthalmoscopy. The number of trapped leukocytes in each group was assessed with a personal computer-based image analysis.

**Results.** Interferon alpha induced leukocyte entrapment in retinal microcirculation and increased leukocyte adherence to the vessel walls. The simultaneous administration of PSL, CV-6209, or SOD inhibited leukocyte adherence to the venous walls and significantly reduced the number of trapped leukocytes.

**Conclusions.** Acridine orange digital fluorography was helpful to quantitate leukocyte-endothelial interactions in retinal microcirculation. The results suggested that increased leukocyte adherence after IFN alpha administration was reduced significantly by PSL, CV-6209, or SOD. These agents may be useful to prevent IFN-induced microcirculatory disturbances. Invest Ophthalmol Vis Sci. 1997;38:811–816.

Recently, interferon (IFN) alpha has been suggested for the treatment of choroidal neovascularization. However, retinal complications as well as systemic side effects have been reported after IFN therapy. Gu yer et al have described IFN-associated retinopathy as focal retinal ischemia including cotton-wool spots, capillary nonperfusion, and retinal hemorrhages. Since Ikebe et al reported a case of IFN-associated retinopathy in 1990, the incidence of reported cases has been increasing because more than 80,000 patients with non-A and non-B hepatitis are treated annually with IFN alpha therapy in Japan. Chuman et al have described 50 consecutive patients receiving IFN therapy; 23 patients (46%) showed retinal complications. The pathogenesis of the retinal complications, however, is unclear.

Previously, we have shown that IFN alpha induced leukocyte adherence to vessel walls and entrapment in retinal microcirculation in rats. Interferon has produced various effects on immunologic cells such as activation of natural killer cells, macrophages, lymphocytes, monocytes, and neutrophils. Interferon has been shown to enhance activation, adherence, and phagocytosis of leukocytes. When leukocytes are
Animal Preparation

Anesthetized animals were catherized through tail veins. A solution of human recombinant IFN alpha-2b (Intron A; Schering, Kenilworth, NJ) in saline was administered with a microinjector (1 X 10⁶ U/kg, 0.1 ml/30 minutes). The dosage of IFN was determined by the previous study in which significant leukocyte adherence to venous walls and entrapment in the retinal microcirculation were observed at that dosage. Saline solution was injected in the same procedure for the control subject. Interferon and the following drug solutions were administered simultaneously through different tail veins. Prednisolone (PSL) (WAKO, Osaka, Japan) was given at a dosage of 1.25 mg/kg as a bolus 10 minutes before IFN administration, followed by an injection of 2.5 mg/kg per 30 minutes. Platelet-activating factor receptor antagonist (CV-6209) (WAKO) was given at a dosage of 0.03 mg/kg as a bolus 10 minutes before IFN administration, followed by an injection of 0.1 mg/kg per 30 minutes. Superoxide dismutase (SOD) (WAKO) was given at a dosage of 3000 U/kg as a bolus 10 minutes before IFN administration, followed by an injection of 12,000 U/kg per 30 minutes. In each group, six rats were used.

Acridine Orange Digital Fluorography

The rat fundus was observed with acridine orange (AO) digital fluorography. This technique has been described previously. We used a fluorescent dye of AO, which is used widely as a probe to show the quantity and conformation of nucleic acids in biochemical and cytochemical studies. The dye fluoresces green when it interacts with double-stranded nucleic acids (DNAs). The spectral properties of AO–DNA complexes are similar to those of fluorescein sodium, with an excitation maximum at 502 nm and an emission maximum at 522 nm. When AO solution is injected intravenously, staining occurs in leukocytes among the circulating blood cells and in nuclei of vascular endothelial cells.

After formulated solutions were administered, AO (0.1% solution in saline; WAKO) was injected continuously for 3 minutes through a tail vein. The total dosage of dye was 10 mg/kg. The retinal images were generated with use of a scanning laser ophthalmoscope (Rodenstock Instrument, Munich, Germany). An argon blue laser was used for the illumination source with a regular emission filter for fluorescein angiography. We observed leukocyte dynamics in the retinal peripapillary area with a 40° field during dye injection and recorded the images for 5 minutes. Thirty minutes after AO was injected, we observed the fundus and recorded the images to analyze leukocyte entrapment in the retina. The obtained images were stored on S-VHS videotape (30 frames/second). Using replayed videotape images, leukocyte dynamics in the retina were studied.

Data Analysis

The video recordings were analyzed by an image analysis system, described in detail elsewhere, consisting of a personal computer equipped with a video digitizer (Radius, San Jose, CA). The latter digitizes the video image in real time (30 frames/second) to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps (8 bits).

The leukocytes trapped in the peripapillary area were counted 30 minutes after AO injection. Eight peripapillary areas surrounded by the adjacent vessels (Fig. 1) were measured in pixels using image software (Image 1.44; National Institutes of Health, Bethesda, MD) and converted to the real value. The observer
FIGURE 1. An observation area is determined by drawing a polygon surrounded by adjacent large vessels as accurately as possible with a cursor. The area is calculated in pixels by image analysis software. Trapped leukocytes are shown as fluorescent dots.

was one of us who did not know the dosage of IFN. A calibration factor was determined to convert the obtained data in pixels to the real value (square millimeters). In brief, three eyes of the rats were enucleated after the experiment, and the real size of each optic disc was measured by microscopy. The size of each optic disc was measured on a computer monitor in pixels using image software. The ratio between the actual size and the apparent value on a computer monitor was calculated. A calibration factor was determined by averaging these ratios. The density of trapped leukocytes was calculated by dividing the number of trapped leukocytes by the surrounding area.

RESULTS

Increased Leukocyte Adherence to Vascular Walls

Circulating leukocytes clearly were observed moving in retinal circulation at the beginning of AO injection. Because AO is membrane permeable, the dye diffused quickly through the blood vessel walls into the retina. One or 2 minutes after the end of dye administration, leukocytes adhering to the vessel walls remained fluorescent in the rats receiving IFN only (Fig. 2), whereas circulating leukocytes showed less fluorescence, probably the result of the washout effect. The fluorescence of the leukocytes adherent to the venous walls faded in approximately 10 minutes. Leukocytes did not appear to adhere to arterial walls. This phenomenon was observed only in the rats receiving IFN alpha at a rate of $1 \times 10^6$ U/kg for 30 minutes. Leukocyte adherence to venous walls was not recognized in the control rats or in those receiving PSL, anti-PAF, or SOD.

FIGURE 2. Leukocytes are observed adhering to the venous wall (for more than 1 minute in video play) in rats receiving interferon alpha at a dosage of $1 \times 10^6$ U/kg (arrow). This finding was not observed in the control subjects or in the groups receiving prednisolone, CV-6209, or superoxide dismutase. This picture was taken 2 minutes after the end of dye injection. The number of fluorescent circulating leukocytes in the vessels was few. The walls of arteries and veins also were fluorescent.

Leukocyte Entrapment in Retinal Microcirculation

Retinal fluorescence decreased gradually after the end of dye injection because of the washout effect. In contrast, trapped leukocytes remained fluorescent for more than 2 hours after dye administration. The number of trapped leukocytes was counted 30 minutes after dye administration. Approximately 30 minutes after the cessation of AO injection, trapped leukocytes clearly were visible, whereas no circulating leukocytes showed fluorescence and retinal fluorescence decreased (Fig. 3). The mean ± standard deviation den-

FIGURE 3. The trapped leukocytes in the rats receiving interferon only ($10^6$ U/kg) were shown as numerous fluorescent dots. This picture was taken 30 minutes after dye injection. Circulating leukocytes and vascular endothelium showed little fluorescence because the dye was washed out.
FIGURE 4. Density of trapped leukocytes is shown. There were significant differences between the control subjects and the group receiving interferon only (1 X 106 U/kg) (P < 0.001, Welch's t-test) and between the group receiving IFN only and each group receiving an agent (prednisolone, CV-6209, or superoxide dismutase) (P < 0.001, Welch's t-test). PSL = prednisolone; CV-6209 = a specific platelet-activating factor receptor antagonist; SOD = superoxide dismutase. Results shown are mean ± standard deviation.

DISCUSSION

Our previous study has shown that IFN alpha increased leukocyte adherence to vessel walls and entrapment in the retinal microcirculation. These results are supported by the evidence that IFN has increased leukocyte adherence and phagocytosis, nitroblue tetrazolium dye reduction, and CD69 expression (a marker of leukocyte activation) and L-selectin homing receptor on the surface of leukocytes. Higuchi et al showed that IFN alpha induced albumin leakage and hemorrhagic changes as well as increased leukocyte adherence to vascular walls in rat mesentery vessels. Many studies have indicated that increased activation and adherence of leukocytes are involved in various pathologic conditions, including hemorrhagic shock, hypertension, and ischemia-reperfusion injury. Leukopenia is observed consistently within hours of exposure during IFN therapy. This finding may be explained by the entrapment of leukocytes in the systemic microcirculation rather than by bone marrow suppression.

This study shows that PSL, PAF receptor antagonist (CV-6209), or SOD significantly reduces leukocyte entrapment induced by IFN alpha. Leukocyte adherence is modulated by the substances produced by leukocytes and endothelial cells. Platelet-activating factor and leukotriene B4 are known to activate leukocytes and endothelial cells and to enhance leukocyte adherence to vascular walls. Platelet-activating factor receptor antagonist has been shown to inhibit leukocyte adherence induced by hydrogen peroxide and ischemia-reperfusion phenomenon. The IFN alpha has been shown to enhance phospholipase A2 activity in bovine endothelial cells. Prednisolone inhibits phospholipase A2, which leads to block PAF synthesis and arachidonic acid metabolites, including LTβ. In response to the activation of leukocytes, leukocytes release both superoxide anion and hydrogen peroxide. Oxygen-derived free radicals are known to enhance leukocyte-endothelial adherence. Superoxide dismutase was shown to inhibit leukocyte adhesion induced by PAF and ischemia-reperfusion. Many studies have shown that leukocyte depletion or prevention of leukocyte adherence attenuated tissue damage in microvascular disturbance models such as phorbol myristate acetate (PMA)-induced lung injury, ischemia-reperfusion myocardial infarction, and circulatory shock.

It has been reported that IFN-associated retinopathy developed from 2 weeks to 4 months after the initiation of IFN therapy. The daily dosage of IFN alpha for the treatment of hepatitis C usually is 105 U/kg. However, our previous study showed that rats receiving 105 U/kg showed significant leukocyte entrapment, as compared with the control subject, which was given only saline. Chuman et al showed that patients with diabetes mellitus, hypertension, retinal arterial sclerosis, and anemia were at risk for IFN retinopathy. Endothelial injury in these diseases may enhance the incidence of IFN retinopathy.

Recently, we have described AO digital fluorography (AODF), which is helpful to investigate leukocyte dynamics in retinal microcirculation in vivo. The results of the previous and current studies showed that AO digital fluorography was a useful tool to study leukocyte adherence to retinal vessels and its entrapment in retinal microcirculation. Circulating leukocytes clearly were visible during AO administra-
Reduction of Interferon-Induced Leukocyte Entrapment

One or 2 minutes after the end of dye administration, leukocytes adhering to the vessel walls remained fluorescent (Fig. 2), whereas circulating leukocytes were less fluorescent, probably because of the washout effect. Observation of trapped leukocytes was possible approximately 2 hours after the end of dye injection without much reduction in fluorescence. This probably is because the dye of trapped leukocytes could not be washed out because they plugged the capillaries. Another possibility is that migrating leukocytes were stained with the dye. However, leukocyte migration usually is accompanied by vascular endothelial changes. Our previous study did not show sodium fluorescein leakage after IFN administration.

Interferon alpha and beta show less species specificity than does IFN gamma. Human IFN alpha has produced antiviral activity in rabbits,5,46 mice,46 and rats.47 Our results showed that the rat retina was sensitive to human recombinant IFN alpha.

In conclusion, our results suggested that increased leukocyte adherence and entrapment induced by IFN alpha administration may cause microcirculatory disturbances of IFN-induced retinopathy. To prevent the retinal complications, some anti-inflammatory drugs that modulate leukocyte-endothelial interactions would be effective.

Key Words

acridine orange digital fluorography, IFN alpha, IFN-induced retinopathy, leukocyte adherence, leukocyte entrapment

References

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