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# Structure of a Porcine Spleen Low-Molecular-Weight Inhibitor of *in Vitro* Colony Formation by Bone Marrow Cells

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The structure of a granulocyte-macrophage colony-inhibitory factor, which was isolated from porcine spleen, was identified as *O*-phosphoethanolamine by means of $^{1}H$- and $^{13}C$-nuclear magnetic resonance, infrared and mass spectroscopic analyses. The biological activity of authentic *O*-phosphoethanolamine was equivalent to that of the purified colony-inhibitory factor, supporting the above conclusion.

**Keywords**——colony-inhibitory factor; colony-forming unit; spleen; bone marrow cell; *O*-phosphoethanolamine; $^{1}H$-NMR; $^{13}C$-NMR

Hematopoietic progenitor cells in mouse bone marrow proliferate and differentiate to form granulocyte-macrophage cell colonies in semi-solid agar culture system.[1,2] It is known that lactoferrin,[3] prostaglandin E,[4] glucocorticoid[5] and serum lipoprotein[6,7] inhibit granulocyte-macrophage colony formation. Peptide inhibitors have also been demonstrated in the spleens of rat and calf.[8,9]

We have found two non-peptide inhibitors in porcine spleen. The apparent molecular weights of these inhibitors were estimated by Bio-Gel P-2 column chromatography to be 300 and 400 daltons, respectively. The specific activity and the content of the 400-dalton species were higher than those of the 300-dalton species in the spleen.[10]

The aim of the present study was to determine the structure of the 400-dalton inhibitor.

## Experimental

**Purification Procedure**——Purification of the inhibitor was performed as previously described.[10] Freshly removed porcine spleens were homogenized with twenty volumes of distilled water (w/v) in a Waring blender at 0 °C. The homogenates were centrifuged at 100000 *g* for 30 min at 4 °C, and the clear supernatants obtained were ultrafiltered on Toyo UK-10 membranes (exclusion limit: 10000 daltons). The ultrafiltrates were concentrated by rotary evaporation.

Step I (Bio-Gel P-2 Column Chromatography): The 50-fold-concentrated ultrafiltrates were applied to a Bio-Gel P-2 column (1.6 × 33 cm) and eluted in 4 ml fractions with distilled water at a flow rate of 0.8 ml/min at 4 °C.

Step II (Silica Gel Column Chromatography): The fractions containing the inhibitor from step I were applied to a silica gel column (1.6 × 6.2 cm) and eluted in 14 g fractions with $CHCl_3$–MeOH–$NH_4OH$ (2 : 2 : 1).

Step III (Paper Electrophoresis): The inhibitor from step II was subjected to paper electrophoresis. The electrophoresis was run on Toyo No. 514 filter paper (10 × 8 cm) at 40 V/cm for 10 min with pyridine–AcOH–$H_2O$ (10 : 0.4 : 90), pH 6.4. The inhibitor was eluted with distilled water.

Step IV (Cellulose Thin Layer Chromatography): The inhibitor from step III was applied on a cellulose thin layer plate (20 × 20 cm, Funakoshi) and developed with *n*-BuOH–AcOH–$H_2O$ (3 : 1 : 1). The inhibitor was eluted with distilled water.

White needle crystals of inhibitor from step IV were obtained by recrystallization from MeOH, and were used for the following measurements of the spectra and melting point. The yield of the final product averaged 74.7 μg/g (wet weight) of spleen.

**Identification of Inhibitor**—Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. The nuclear magnetic resonance (NMR) spectra in $D_2O$ containing sodium 3-(trimethylsilyl)-propanesulfonate as an internal standard were measured on a JEOL JNM-FX 100 spectrometer (99.6 MHz for $^1$H-NMR and 25.05 MHz for $^{13}$C-NMR), and chemical shifts are given in $\delta$ (ppm). Infrared (IR) spectra for KBr disks were recorded on a JASCO A-102 spectrometer. Field desorption-mass spectra (FD-MS) were recorded on a JEOL DX-300 spectrometer.

**Determination of Inhibitory Activity**——Inhibitory activity was determined in a semi-solid agar culture system according to the method previously described.[10] In brief, femoral bone marrow cells ($1 \times 10^5$/ml) of ddY male mice were cultured in 35 mm plastic Petri dishes (Lux) in 1.0 ml of the nutrient mixture F-10 medium (Gibco) containing 0.35% Bacto-agar (Difco), 20% calf serum (Gibco) and 15% L-cell conditioned medium as a source of colony-stimulating factor. The inhibitor was added to the culture system in a volume of 0.1 ml. After 8 d of incubation at 37 °C in a fully humidified atmosphere of 5% $CO_2$ in air, discrete colonies containing more than 50 cells were counted with a dissecting microscope. Inhibitory activity is represented as percent reduction in colony number.

## Results and Discussion

### Identification of the Inhibitor

The inhibitor was recrystallized from methanol to yield needles; mp 231.5—234 °C. The $^1$H-NMR spectrum showed two methylene proton signals at $\delta$ 4.09 (2H, dt, $J$=6.4, 5 Hz) and $\delta$ 3.26 (2H, t, $J$=5 Hz). This spectrum was similar to that of ethanolamine in both the chemical shifts and signal pattern. In order to confirm that these methylene proton signals were coupled to each other, we observed changes of the signal patterns caused by the use of the spin–spin decoupling technique. Decoupling of the proton at $\delta$ 4.09 caused the triplet (5 Hz) at $\delta$ 3.26 to collapse to a broad singlet, and decoupling the protons at $\delta$ 3.26 caused the doublet of triplets ($J$=6.4, 5 Hz) to collapse to two peaks ($J$=6.4 Hz). On the basis of these spectral data it is concluded that these two methylene proton signals were coupled to each other with $J$=5 Hz, and the signals at $\delta$ 4.09 (d, $J$=6.4 Hz) were assigned to protons coupled to a nucleus other than a proton. On the other hand, under complete proton noise decoupling conditions, the $^{13}$C-NMR spectrum showed two doublet signals at $\delta$ 64.1 ($J$=5 Hz) and at $\delta$ 42.7 ($J$=8 Hz, long-range) coupled with a nucleus of spin quantum number $I$=1/2, other than a proton, as in the case of the $^1$H-NMR spectrum. The C–H coupling constants of the lowfield and highfield signals, which were measured in the nuclear Overhauser effect (NOE) mode without decoupling, were 146 and 142 Hz, respectively, at each of the triplets. The signal at $\delta$ 64.1 was assigned to the methylene-carbon of P–O–$CH_2$, and the signal at $\delta$ 42.7 was assigned to the methylene-carbon adjacent to an amino group. The $^1$H-NMR and $^{13}$C-NMR spectral data indicate a structure for the inhibitor in which a $^{31}$P-nucleus of spin quantam number $I$=1/2 is bound to ethanolamine through an oxygen atom.

The IR spectrum showed absorptions at 2920, 1640, 1555, 1259, 1162, 1098, 1083, 1035, 945 and 768 $cm^{-1}$. The FD-MS spectrum showed a molecular ion peak at $m/z$ 142 $(M+H)^+$.

Table I. Inhibitory Activities of PEA and the Purified Inhibitor

| Concentration ($\mu$g/ml) | Inhibitory activity (% inhibition)[a] | |
|---|---|---|
| | PEA | Purified inhibitor |
| 4.55 | 1.1±5.1 | 1.0±3.5 |
| 9.09 | 19.2±4.7 | 13.5±6.5 |
| 13.64 | 27.9±4.5 | 28.9±3.7 |
| 18.18 | 68.4±8.8 | 67.6±9.6 |
| 22.73 | 95.2±2.5 | 93.7±1.5 |
| 27.27 | 100 | 100 |

a) Means of six replicate cultures ± S.D. Control colony counts were 112.2±3.2.

On the basis of these spectral data it was considered that the inhibitor is *O*-phosphoethanolamine (PEA). Authentic PEA (Calbiochem-Behring) showed mp 231.5—234 °C,[11] and the mixed melting point showed no depression. Further, the IR, $^{1}H$- and $^{13}C$-NMR, and FD-MS spectra of the inhibitor were the same as those of PEA. Thus, the inhibitor was concluded to be PEA.

**Inhibitory Activity of PEA**

Biological assay was also carried out to compare the inhibitory activities of the inhibitor and PEA. The inhibitory activity of PEA was equivalent to that of the purified inhibitor (as seen in Table I), supporting the above conclusion that the inhibitor is PEA. PEA is a precursor of phospholipids (phosphatidylethanolamine and phosphatidylcholine), so the inhibitory effect of the inhibitor may arise from an effect on phospholipid synthesis.

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