Effects of DNA on immunoglobulin production stimulating activity of alcohol dehydrogenase

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Abstract

Alcohol dehydrogenase-I (ADH-I) derived from horse liver stimulated IgM production by human-human hybridoma, HB4C5 cells and lymphocytes. The IPSF activity of ADH-I was suppressed by coexistence of short DNA whose chain length is less than 200 base pairs (bp) and fibrous DNA in a dose-dependent manner. These DNA preparations completely inhibited the IPSF activity at the concentration of 250 μ g/ml and 1.0 mg/ml, respectively. DNA sample termed long DNA whose average chain length is 400–7000 bp slightly stimulated IPSF activity at 0.06 μ g/ml. However, long DNA suppressed IPSF activity by half at 1.0 mg/ml. The laser confocal microscopic analysis had revealed that ADH-I was incorporated by HB4C5 cells. The uptake of ADH-I was strongly inhibited by short DNA and fibrous DNA. However, long DNA did not suppress the internalization of ADH-I into HB4C5 cells. These findings indicate that short DNA and fibrous DNA depress IPSF activity of ADH-I by inhibiting the internalization of this enzyme. According to the gel-filtration analysis using HPLC, ADH-I did not directly interact with short DNA. It is expected from these findings that short DNA influences HB4C5 cells to suppress the internalization of ADH-I. Moreover, these facts also strongly suggest that ADH-I acts as IPSF after internalization into the cell.

Abbreviations: ADH, alcohol dehydrogenase; BSA, bovine serum albumin; DNA, deoxyribonucleic acid; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ig, immunoglobulin; IPSF, immunoglobulin production stimulating factor; MAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PBS, phosphate buffered saline; PI, propidium iodide

Introduction

Monoclonal antibodies (MAbs) are applied to various field, such as therapy, diagnosis, immunohistochemistry and also used for industrial applications to purify products by immunoaffinity chromatography. Increased demands for MAbs have prompted attention to improve the cellular productivity of hybridomas, especially human-human hybridomas. There are two strategies to produce MAbs in large quantities under serum-free conditions. One is high-density large-scale culture of hybridomas, and the other is the enhancement of immunoglobulin (Ig) productivity

of hybridomas. We have demonstrated that there are substances termed Ig production stimulating factors (IPSF) which enhance Ig production by hybridomas and lymphocytes.

We previously reported that alcohol dehydrogenase (EC1.1.1.1; ADH) was identified as IPSF (Sugahara et al., 1997). ADH-I derived from horse liver stimulated IgM production by human-human hybridoma, HB4C5 cells producing human lung cancer specific monoclonal IgM. IgM production of HB4C5 cells was enhanced more than 8.5-fold by the addition of ADH-I under serum-free condition. However, yeast derived ADHs, such as ADH-II and ADH-III were in-

effective to accelerate Ig production by the hybridoma line. This result revealed that the Ig production stimulating effect of ADH-I was irrelevant to its enzymatic function, and was defined as a novel feature of ADH-I. This enzyme also stimulated IgM and IgG production by human peripheral blood lymphocytes (PBL) 2.9- and 1.4-fold, respectively. This fact suggests that ADH-I stimulates Ig production not only by specific hybridoma cell line, but also by non-specific Ig producers.

Our previous data indicated that coexistence of nucleic acids influenced on activity of some IPSFs. IPSF activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was inactivated by the coexistence of DNA, synthetic polyribonucleic acids, and NAD^+ in the medium (Sugahara et al., 1998). On the other hand, IPSF activity of spermine was activated by DNA 2-fold (Miyazaki et al., 1998). In this paper, we report the effects of DNA on Ig production stimulating activity of ADH-I from horse liver.

Materials and methods

Alcohol dehydrogenase

ADH-I from horse liver was purchased from Boehringer Mannheim, (Germany). ADH-I was dialyzed against 10 mM sodium phosphate buffer (pH 7.4) and sterilized by filtration before use. The ADH-I preparation was gel-filtrated to confirm its purity. As the result of gel-filtration using HPLC, only single peak was detected by measuring absorbance at 280 nm, and the SDS-PAGE analysis proved that the peak fraction contained only ADH-I protein and the IPSF activity was derived from ADH-I (Sugahara et al., 1997).

Deoxyribonucleic acids

Three DNA products were used here. Degraded DNA from herring sperm whose chain length is less than 200 base pairs (bp) was purchased from Sigma (USA). DNA from salmon sperm (chain length: 400–7000 bp) and fibrous DNA from salmon sperm (molecular weight: 20 M–30 MDa) were from Wako Pure Chemistry (Japan). In this report, these DNA preparations were termed as short DNA (<200 bp), long DNA (400–7000 bp) and fibrous DNA (20 M–30 MDa), respectively. Each DNA preparation was dissolved in 10 mM sodium phosphate buffer (pH

7.4). Before usage, the DNA solutions were boiled for 5 min and placed on ice.

Cell and cell culture

Human-human hybridoma HB4C5 cell producing monoclonal IgM was used to assay the IPSF activity of ADH-I. HB4C5 cells were fusion product of a human lymphocyte from lung cancer patient and a human fusion partner, NAT-30 (Murakami et al., 1985). HB4C5 cells were cultured in ERDF medium (Kyokuto Pharmaceutical, Japan) supplemented with 10 $\mu\text{g}/\text{ml}$ of insulin, 20 $\mu\text{g}/\text{ml}$ of transferrin, 20 μM of ethanolamine and 25 nM of selenite (ITES-ERDF) at 37 °C under humidified 5% CO_2 -95% air (Murakami et al., 1982).

Assay of the IPSF activity of ADH-I

The IPSF activity of ADH-I was examined by measuring the amount of antibody secreted by HB4C5 cells in ITES-ERDF medium. The assay of the IPSF activity was performed in a 96-well culture plate of which culture volume is 200 $\mu\text{l}/\text{well}$. HB4C5 cells were inoculated at 1×10^5 cells/ml. After cultivation in CO_2 -incubator at 37 °C for 6 h, the amount of antibody secreted in each culture medium was determined by enzyme-linked immunosorbent assay (ELISA) using anti-human IgM antibody (TAGO, USA) as mentioned in previous report (Sugahara et al., 1994).

Laser confocal microscopic analysis of the uptake of ADH-I by HB4C5 cells

ADH-I was directly labeled by FITC using FluoroTag FITC conjugation kit purchased from Sigma (USA). Following the labeling reaction, FITC-conjugated ADH-I was separated from the unconjugated FITC by Sephadex G-25 column (Pharmacia, Sweden). HB4C5 cells were cultured in ITES-ERDF medium supplemented with FITC-conjugated ADH-I in a CO_2 -incubator at 37 °C. After cultivation, HB4C5 cells were washed 3 times with phosphate buffered saline (PBS), fixed for 10 min at room temperature with 95% ethanol, and mounted on slides using Permafluor (Immunotech S.A., France) containing 50% glycerol. Samples were visualized by a laser scanning confocal microscope with an argon ion laser (488 nm) coupled with Meridian InSIGHT plus confocal unit attached to Olympus IMT-2 inverted research.

Figure 1. Effect of short DNA on IPSF activity of ADH-I. Short DNA from herring sperm was mixed with the same volume of ADH-I (●) or not (○) at various concentrations and incubated at 37 °C for 2 h. Following incubation, the mixture was added to ITES-ERDF medium to confirm the IPSF activity of ADH-I. The final concentration of ADH-I in the medium was 800 $\mu\text{g/ml}$. After cultivation for 6 h, the amount of IgM in culture medium was measured by ELISA. Relative IPSF activity means the IPSF effect of ADH-I treated with short DNA against IgM production in ITES-ERDF medium supplemented with each concentration of short DNA. Results are expressed as the means $\pm$ S.D. of three independent measurements.

Result

Effect of short DNA on IPSF activity of ADH-I

The effect of short DNA on IPSF activity of ADH-I was examined. HB4C5 cells were inoculated in ITES-ERDF medium containing 800 $\mu\text{g/ml}$ of ADH-I and various concentrations of short DNA. The cells were cultured for 6 h. As indicated in Figure 1, ADH-I facilitated IgM production by HB4C5 cells 3.0-fold when short DNA was absent, and short DNA did not affect IgM production by the hybridoma line at any dose. However, the IPSF activity of ADH-I was depressed by the coexistence of short DNA in a dose dependent

Figure 2. Effect of long DNA on IPSF activity of ADH-I. Long DNA from salmon spermary was mixed with the same volume of ADH-I (●) or not (○) at various concentrations and incubated at 37 °C for 2 h. HB4C5 cells were inoculated at 1×10^5 cells/ml in ITES-ERDF media containing the mixture. The final concentration of ADH-I was 800 $\mu\text{g/ml}$. After cultivation for 6 h, the amount of IgM in culture medium was measured by ELISA. Relative IPSF activity means the IPSF effect of ADH-I treated with long DNA against IgM production in ITES-ERDF medium supplemented with each concentration of long DNA. Results are expressed as the means $\pm$ S.D. of three independent measurements.

manner above 1 $\mu\text{g/ml}$. Finally, the amount of IgM in the ITES-ERDF medium supplemented with ADH-I was decreased to the control level by 250 $\mu\text{g/ml}$ of short DNA. This result indicated that the IPSF effect of this enzyme completely disappeared.

Effect of long DNA on IPSF activity of ADH-I

The effect of long DNA on IPSF activity of ADH-I was determined. As well as short DNA, long DNA itself had no influence on IgM production by HB4C5 cells at any concentrations (Figure 2). The addition of lower concentrations of long DNA weakly promoted the IPSF activity of ADH-I. The IPSF activity was improved 1.4-fold at 0.06 $\mu\text{g/ml}$ of long DNA. However, the effect of ADH-I was slightly inhibited at the higher

Figure 3. Effect of fibrous DNA on IPSF activity of ADH-I. Fibrous DNA from salmon spermary was mixed with the same volume of ADH-I (●) or not (○) at various concentrations and incubated at 37 °C for 2 h. Following incubation, the mixture was added to ITES-ERDF medium to confirm the IPSF activity of ADH-I. The final concentration of ADH-I was 800 μg/ml. After cultivation for 6 h, the amount of IgM in culture medium was measured by ELISA. Relative IPSF activity means the IPSF effect of ADH-I treated with fibrous DNA against IgM production in ITES-ERDF medium supplemented with each concentration of fibrous DNA. Results are expressed as the means ± S.D. of three independent measurements.

concentrations of long DNA. The enzyme retained its IPSF function about 50% at 1.0 mg/ml of long DNA.

Effect of fibrous DNA on IPSF activity of ADH-I

Moreover, the effect of fibrous DNA on the IPSF activity was investigated. Fibrous DNA enhanced IgM production by HB4C5 cells 1.2-fold at 250 μg/ml. In spite of slight stimulation of IgM production by fibrous DNA at the higher concentrations, the IPSF effect of ADH-I was gradually suppressed by coexistence of fibrous DNA (Figure 3). The IPSF effect was completely suppressed by the coexistence of 1.0 mg/ml of fibrous DNA.

Figure 4. Interaction of ADH-I with short DNA. The mixture of ADH-I and Short DNA were incubated for 2 h at 37 °C. After incubation, the mixture was gel-filtrated by Showdex KW-803 column (Showa denko, Japan) with 10 mM sodium phosphate buffer. The elution of the mixture was monitored by absorbance at 280 nm. Short DNA and ADH-I were also gel-filtrated under the same condition. The elution of short DNA and ADH-I was monitored by absorbance at 260 nm and 280 nm, respectively.

Assay of interaction of ADH-I with short DNA

It was clearly demonstrated that short DNA inhibited the IPSF activity of ADH-I in a dose-dependent manner. Then, an direct interaction of ADH-I with short DNA was examined by gel-filtration using HPLC. Following equilibrium of PROTEIN KW-803 column (8.0 mm i.d. $\times$ 300 mm, Showa Denko, Japan) with 10 mM sodium phosphate buffer (pH 7.4), the mixture of ADH-I and short DNA was gel-filtrated. As shown in Figure 4, two peaks were detected by measuring absorbance at 280 nm. The retention time of each peak was 9.7 and 11.0 min, respectively. The retention time of the first peak was consistent with that of short DNA (9.7 min). On the other hand, the retention time of the second peak was consistent with that of ADH-I (11.0 min). The peak of complex of ADH-I and short DNA was not observed. Furthermore, the second peak exclusively retained ADH activity (data not shown). These results indicate that the first peak of the mixture contains only short DNA, and the second one consists of ADH-I. This result suggests that ADH-I does not directly interact with short DNA.

Effects of DNA on internalization of ADH-I into HB4C5 cells

The internalization of ADH-I in HB4C5 cells was investigated. ADH-I directly labeled with FITC was used here. HB4C5 cells were treated with 800 μ g/ml of FITC-conjugated ADH-I for 6 h. Following washing and fixation, the cells were visualized by a laser scanning confocal microscope with an argon ion laser (488 nm). As shown in Figure 5A, HB4C5 cells were strongly stained with FITC-conjugated ADH-I both cell surface and intracellular organelles. However, fluorescence intensity was decreased by the coexistence of 1.0 mg/ml of short DNA at which the IPSF activity of ADH-I is completely suppressed (Figure 5B). As contrasted with this result, 1.0 mg/ml of long DNA did not affect the internalization of ADH-I into HB4C5 cells (Figure 5C).

In addition, a similar investigation was done for the uptake of ADH-I by HB4C5 cells under the coexistence of fibrous DNA. As shown in Figure 6, it was confirmed that the internalization of ADH-I was clearly suppressed by coexistence of 1.0 mg/ml of fibrous DNA. Fibrous DNA was also labeled with propidium iodide (PI) before inoculation. Fibrous DNA was mixed with 1.0 mg/ml of PI and gel-filtrated by Sephadex G-25 column. Fibrous DNA was also internalized by HB4C5 cells (Figure 7A). However,

the uptake of fibrous DNA was not affected by coexistence of 800 μ g/ml of ADH-I (Figure 7B). This fact indicates that the internalization of ADH-I into HB4C5 cells is not competitive with that of fibrous DNA.

These findings suggest that short DNA and fibrous DNA suppress IPSF effect of ADH-I by depressing the internalization of this enzyme into the hybridoma. This fact also implies that ADH-I acts as IPSF after internalization into HB4C5 cells.

Discussion

IPSF stimulates Ig production by hybridomas and lymphocytes. We previously demonstrated that GAPDH was identified as IPSF contained in the cell lysate of human Burkitt's lymphoma, Namalwa cells (Sugahara et al., 1991). GAPDH facilitated Ig production by accelerating post-translational activity of hybridoma cells (Sugahara et al., 1995). The IPSF activity was irrelevant to this enzymatic activity in glycolysis. Hence, the IPSF function was regarded as a novel activity of this enzyme. GAPDH has also been studied by many workers for its functions other than enzymatic activity (Sabath et al., 1990, Nagy et al., 1995 and Singh et al., 1995). GAPDH is a protein having a binding affinity with nucleic acids, such as single stranded DNA, RNA, and mono- and polyribosomes (Perucho et al., 1977, Perucho et al., 1980, and Ryazanov et al., 1988). Moreover, GAPDH is a one of the three major RNA binding proteins in rabbit reticulocyte lysate (Ryazanov, 1985). Therefore, we have screened IPSF on the basis of nucleic acid binding property. As the result of that, lysine-rich histons (H1, H2A and H2B) and poly-lysine were identified as IPSF (Sugahara et al., 1994). Moreover, it was revealed that spermine, a sort of polyamine, also stimulated Ig production by hybridoma HB4C5 cells (Miyazaki et al., 1998). Spermine accelerated IgM productivity of HB4C5 cells 7.7-fold at 2.4 mM. Then we investigated the effect of nucleic acids on the IPSF activity of nucleic acid binding substances. The IPSF effect of GAPDH was suppressed by the coexistence with DNA, synthetic polyribonucleic acids, and NAD^+ (Sugahara et al., 1998). On the contrary, IPSF activity of spermine was enhanced 2-fold by coexistence of DNA in the medium.

On the other hand, we previously reported that ADH-I from horse liver facilitated Ig production by HB4C5 cells and human PBL under serum-free con-

Figure 5. Effects of short and long DNA on internalization of ADH-I into HB4C5 cells. HB4C5 cells were inoculated at 2×10^6 cells/ml in ITES-ERDF medium containing $800 \mu\text{g/ml}$ of FITC-conjugated ADH-I and 1.0 mg/ml of short and long DNA preparations, and cultured for 6 h. After cultivation, these cells were washed 3 times and fixed with 95% ethanol. Fluorescence derived from FITC was monitored by a laser scanning confocal microscope under the equal condition. [A]; without DNA (as control), [B]; with short DNA, [C]; long DNA.

Figure 6. Effect of fibrous DNA on internalization of ADH-I into HB4C5 cells. HB4C5 cells were inoculated at 2×10^6 cells/ml in ITES-ERDF medium containing $800 \mu\text{g/ml}$ of FITC-conjugated ADH-I coexistence with [B] or without [A] 1.0 mg/ml of fibrous DNA. After 6-h cultivation, these cells were washed 3 times and fixed with 95% ethanol. Fluorescence of FITC was monitored by a laser scanning confocal microscope under the equal condition.

ditions (Sugahara et al., 1997). The effect of DNA on IPSF activity of ADH-I was demonstrated here. The IPSF activity of ADH-I was suppressed by short DNA whose chain length is less than 200 bp at $250 \mu\text{g/ml}$, as well as that of GAPDH. Fibrous DNA also depress the effect of ADH-I. In contrast to these results, long DNA of 400–7000 bp facilitated IPSF activity at $0.06 \mu\text{g/ml}$. It is obvious from these findings that the length of DNA is not important for the inhibition activity. In addition, the origin of DNA is also independent from the inhibition activity, because long DNA and fibrous

DNA came from same origin.

The inhibition effects of polyribonucleotides were investigated. As the result of that, polyinosinate:polycytidylate [Poly (I):poly(C)], double-stranded polyribonucleotides, and tRNA suppressed the IPSF activity of ADH-I. Polyuridylate [Poly (U)] depressed the IPSF activity of ADH-I by half. However, polyadenylate [Poly (A)] and polycytidylate [Poly (C)] were ineffective to inhibit the IPSF activity (data not shown). It is expected from these findings that base components of nucleic acid are related to the inhibition

Figure 7. Internalization of fibrous DNA into HB4C5 cells under coexistence of ADH-I. HB4C5 cells were inoculated at 2×10^6 cells/ml in ITES-ERDF medium containing 1.0 mg/ml of PI-conjugated fibrous DNA coexistence with [B] or without [A] 800 μ g/ml of ADH-I. After 6-h cultivation, these cells were washed 3 times and fixed with 95% ethanol. Fluorescence of PI was monitored by a laser scanning confocal microscope under the equal condition.

effects on the IPSF activity of ADH-I.

According to the gel-filtration analysis using HPLC, ADH-I, however, did not directly interact with short DNA which completely inhibited the IPSF activity. Laser confocal microscopic analysis revealed that ADH-I was incorporated by HB4C5 cells, and the internalization was suppressed by the coexistence of short DNA or fibrous DNA, not by long DNA. These results imply that short DNA suppresses internalization of ADH-I by affecting not ADH-I but HB4C5 cells. This fact also strongly suggested that ADH-I acts as IPSF after internalization into the cell. It is still not clear how ADH-I facilitates Ig production by hybridomas and lymphocytes. Discovering the mode of action of ADH-I will contribute to the effective production of Ig by animal cells.

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