Characterization of human foetal intestinal alkaline phosphatase

Comparison with the isoenzymes from the adult intestine and human tumour cell lines

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The molecular structure of human foetal intestinal alkaline phosphatase was defined by high-resolution two-dimensional polyacrylamide-gel electrophoresis and amino acid inhibition studies. Comparison was made with the adult form of intestinal alkaline phosphatase, as well as with alkaline phosphatases isolated from cultured foetal amnion cells (FL) and a human tumour cell line (KB). Two non-identical subunits were isolated from the foetal intestinal isoenzyme, one having the same molecular weight and isoelectric point as placental alkaline phosphatase, and the other corresponding to a glycosylated subunit of the adult intestinal enzyme. The FL-cell and KB-cell alkaline phosphatases were also found to contain two subunits similar to those of the foetal intestinal isoenzyme. Characterization of neuraminidase digests of the non-placental subunit showed it to be indistinguishable from the subunits of the adult intestinal isoenzyme. This implies that no new phosphatase structural gene is involved in the transition from the expression of foetal to adult intestinal alkaline phosphatase, but that the molecular changes involve suppression of the placental subunit and loss of neuraminic acid from the non-placental subunit. Enzyme-inhibition studies demonstrated an intermediate response to the inhibitors tested for the foetal intestinal, FL-cell and KB-cell isoenzymes when compared with the placental, adult intestinal and liver forms. This result is consistent with the mixed-subunit structure observed for the former set of isoenzymes. In summary, this study has defined the molecular subunit structure of the foetal intestinal form of alkaline phosphatase and has demonstrated its expression in a human tumour cell line.

The human alkaline phosphatases (EC 3.1.3.1) exist in a number of distinct molecular forms, which are specific for various tissues and cultured cell lines (Gottlieb & Sussman, 1968; Harkness, 1968; Suzuki *et al.*, 1975; Ludueña & Sussman, 1976; Badger & Sussman, 1976; Hamilton *et al.*, 1979a,b; McKenna *et al.*, 1979). Three distinct classes of alkaline phosphatases have been identified by differences in protein structure as determined by peptide 'maps', partial sequence analysis and immunological analysis. These classes of alkaline phosphatase are those characteristic of (1) term placenta, (2) adult intestine and (3) liver, kidney and bone. Because each of these isoenzymes is a homodimer, it has been suggested that the synthesis of each is coded by a separate gene or genetic locus (Mulivor *et al.*, 1978a; McKenna *et al.*, 1979; Seargeant & Stinson,

1979; Whitaker & Moss, 1979). A variant subunit has been shown in KB cells. It is co-precipitated with the term-placental subunit by polyclonal antiserum to term-placental alkaline phosphatase, but has a different surface charge, molecular weight and *N*-terminal residue (Ludueña & Sussman, 1976). A subunit in cloned Chang liver cells, of similar molecular weight and surface charge to the variant subunit, was co-precipitated with the placental subunit by the same anti-(placental alkaline phosphatase) serum (Hamilton *et al.*, 1979a).

Other investigations have also confirmed the existence of a foetal intestinal alkaline phosphatase that is distinctively different from the adult intestinal enzyme (Suzuki *et al.*, 1975; Mulivor *et al.*, 1978a; Miki *et al.*, 1978; Gogolin *et al.*, 1982). An alkaline phosphatase with electrophoretic mobility similar to that of foetal intestinal alkaline phosphatase has been observed in hepatoma cells (Suzuki

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et al., 1975) and in FL cells, a cell line cultured from the foetal amnion (Higashino *et al.*, 1975). The presence of the foetal intestinal enzyme in cell lines derived from neoplastic tissue is consistent with its being ectopically expressed by neoplastic cells, as has been established for the term-placental enzyme (Fishman *et al.*, 1968; Nathanson & Fishman, 1971; Sussman *et al.*, 1974). Although the existence of a foetal intestinal alkaline phosphatase has been identified, the nature of its structure has not been determined, and conflicting reports have been presented (Miki *et al.*, 1978; Mulivor *et al.*, 1978a).

We have undertaken this present study to clarify the molecular nature of the structural difference between the foetal and the adult intestinal alkaline phosphatases as well as to determine whether this difference can be attributed to the existence of unique genotypes. Structural comparisons were made by utilizing high-resolution two-dimensional polyacrylamide-gel electrophoresis and enzyme-inhibition studies. In addition, the subunit structure of foetal intestinal alkaline phosphatase was also directly compared with the subunit structure of the alkaline phosphatases obtained from FL and KB cells.

Materials and methods

Preparation of alkaline phosphatase

Human adult intestinal and placental alkaline phosphatases were isolated and purified as described previously (McKenna *et al.*, 1979). For the preparation of foetal intestinal alkaline phosphatase, duodenal and jejunal tissue was obtained from four induced elective abortions performed at 15–18 weeks' gestation. The luminal surface of the intestine was washed with 0.15 M-NaCl; the mucosal cells were then scraped into 10 mM-Tris/HCl buffer, pH 7.4, and washed three times with the same buffer. Alkaline phosphatase was extracted by homogenizing the intestinal mucosal cells in 20% (v/v) butan-1-ol and incubating the homogenate at 37°C with gentle agitation for 2 h (Ludueña & Sussman, 1976). The homogenate was centrifuged at 6000 g for 15 min to separate the alkaline-phosphatase-containing aqueous layer; the butanol phase was re-homogenized and re-extracted with 20% butanol. The pooled aqueous phases were then subjected to fractional acetone precipitation as described by Sussman *et al.* (1968); the acetone precipitate was resuspended in 10 mM-Tris/HCl buffer, pH 7.4, and dialysed extensively against that buffer.

FL (Fogh-Lund) human amniotic membrane cells were obtained from the American Tissue Culture Collection (A.T.C.C. CCL62) and were grown in Dulbecco's Minimal Essential Medium with 10% (v/v) foetal-calf serum and 1% antibiotics/fungicides. KB cells (A.T.C.C. CCL17) were

obtained from Dr. David Korn, Stanford University, and were also grown in Dulbecco's Minimal Essential Medium with 10% foetal-calf serum and 1% antibiotics/fungicides. All media, sera and antibiotics were obtained from Grand Island Biological Co. (Grand Island, NY, U.S.A.).

Final purification of all alkaline phosphatases was accomplished by immunoprecipitation with antibodies raised in sheep against placental alkaline phosphatase, as described previously (McKenna *et al.*, 1979). At least 90% of the alkaline phosphatase activity isolated from each of the tissues could be precipitated with antibodies against placental alkaline phosphatase, indicating a high degree of molecular homogeneity among the alkaline phosphatases studied in this present investigation.

Alkaline phosphatase activity was assayed by the method of Bessey *et al.* (1946), with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) as substrate. All incubations were performed at 37°C in 0.1 M-ethanolamine, pH 10.0; reactions were terminated by the addition of 0.1 M-NaOH. Absorbance was measured at 405 nm in a Brinkmann spectrophotometer; 1 unit of enzyme activity hydrolysed 1 μ mol of *p*-nitrophenyl phosphate/min at 37°C.

Radioimmunoassay of alkaline phosphatase

A double-antibody radioimmunoassay method, which has already been described in detail (Wada *et al.*, 1979), was used to measure the alkaline phosphatases. The method employs high-titre antibody raised in goats to purified human term-placental alkaline phosphatase, and antigen iodinated with 125 I by the use of chloramine-T. Maximal sensitivity is obtained by using antigen and antibody concentrations to give 30% bound antigen. The minimum detectable amount of the placental alkaline phosphatase used in the standard curve was 0.5 ng.

Radioactive labelling and two-dimensional polyacrylamide-gel electrophoresis

After immunoprecipitation with antibody to placental alkaline phosphatase, approx. 0.01 unit of alkaline phosphatase activity was labelled with [32 P]orthophosphoric acid (New England Nuclear) as described previously (Hamilton *et al.*, 1979b). Neuraminidase digestion of some samples was accomplished by incubation with 0.21 unit of neuraminidase (Sigma)/ml in 50 mM-sodium acetate buffer, pH 5.0, for 15 min at 37°C before immunoprecipitation. Two-dimensional electrophoresis of labelled samples was achieved by sequential isoelectric focusing and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in accordance with the O'Farrell (1975) procedure, with modifications suggested by Wada *et al.* (1977). Isoelectric focusing was achieved with a pH gradient

from 3 to 10, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on 3% stacking with 8% separating polyacrylamide gels. All reagents for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and were electrophoresis grade; Ampholines were a product of LKB Produkter (Uppsala, Sweden). The pH gradient of the focusing gels was determined by slicing gels in 0.5 cm segments, incubating each in 1 ml of doubly deionized water at room temperature for 24 h, and measuring the pH with a Corning glass electrode. Gels were stained for protein with Coomassie Blue in accordance with the procedure of Fairbanks *et al.* (1971). Purified unlabelled placental alkaline phosphatase (approx. 10 μ g) with subunit pI 6.0 and M_r 64 000 (McKenna *et al.*, 1979) was added to each gel as an internal standard.

Inhibition studies

Alkaline phosphatase activity was measured in the presence of various inhibitors capable of distinguishing different classes of alkaline phosphatases, and was compared with that of control samples containing no inhibitor. In all determinations, approx. 0.02 unit of enzyme activity was present. Assays were performed in duplicates, which were found consistently to differ by less than 5%.

The inhibitors L-phenylalanine, homoarginine and phenylalanylglycylglycine were products of Sigma; levamisole was purchased from Pitman-Moore (Washington Crossing, NJ, U.S.A.).

Results and discussion

Fig. 1 shows the comparison of the two-dimensional electrophoretic patterns of the various denatured alkaline phosphatase subunits that had been labelled with [32 P]orthophosphoric acid as described in the Materials and methods section. It is readily apparent that foetal intestinal alkaline phosphatase contains two distinct subunits, one with isoelectric point (pI) 6.0 and apparent molecular weight (M_r) approx. 64 000, the other demonstrating pI 5.5 and M_r 72 000. Inclusion of unlabelled placental enzyme as an internal standard on each two-dimensional gel indicated that the foetal intestinal enzyme subunit with pI 6.0 and M_r 64 000 corresponded precisely to the subunits of the placental enzyme. By comparison, the adult intestinal isoenzyme was found to contain a single subunit with pI 5.9 and M_r 70 000; this is in exact agreement with the results reported by McKenna *et al.* (1979). Fig. 1 also shows the patterns obtained with alkaline phosphatase isolated from KB and FL cells; again, it can be seen that

Fig. 1. Two-dimensional sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic patterns of 32 P-labelled alkaline phosphatase isolated from various tissues

Foetal intestinal (a), KB-cell (b), adult intestine (c) and FL-cell (d) alkaline phosphatases were investigated. Isolation, radioactive labelling and electrophoresis were performed as described in the Materials and methods section. The pH gradient for the isoelectric-focusing dimension was measured at 0.5 cm intervals and was determined to be as follows: 8.2, 7.7, 7.6, 7.4, 7.3, 7.2, 7.2, 7.1, 7.1, 7.0, 6.9, 6.8, 6.6, 6.5, 6.0, 5.5, 5.0, 4.7, 4.1, 3.5. Abbreviations: IEF, isoelectric focusing; SDS, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

both alkaline phosphatases contain two subunits that were identical in pI and M_r with the two subunits of the foetal intestinal alkaline phosphatase.

To determine whether the more-acidic, higher-molecular-weight, subunit expressed in foetal intestinal, FL-cell and KB-cell alkaline phosphatases represented a unique gene product, the various isoenzymes were subjected to neuraminidase digestion, as described in the Materials and methods section. Table 1 indicates that, after the removal of sialic acid, the placental-like component of foetal intestinal alkaline phosphatase becomes more basic, as expected from the results obtained by McKenna *et al.* (1979); in addition, the acidic subunit becomes

indistinguishable from the subunits of the adult intestinal enzyme in the two-dimensional electrophoretic separation. These results suggest that the larger, more-acidic, component of the foetal intestinal enzyme represents a glycosylated variant of the adult intestinal alkaline phosphatase. In similar experiments neuraminidase digests of FL-cell and KB-cell alkaline phosphatases gave electrophoretic patterns indistinguishable from that of the foetal intestinal enzyme, i.e. one subunit corresponds to that of placental alkaline phosphatase and the other to a glycosylated subunit of adult alkaline phosphatase and the other to a glycosylated subunit of adult intestinal alkaline phosphatase.

The results of the inhibition studies with foetal intestinal, FL-cell and KB-cell alkaline phosphatases are presented in Table 2. Term-placental alkaline phosphatase activity is markedly inhibited in the presence of L-phenylalanine (Fishman & Sie, 1971). The comparable inhibition of foetal intestinal, FL-cell and KB-cell alkaline phosphatases is consistent with the immunochemical cross-reactivity of these alkaline phosphatases to the placental enzyme and the demonstration that each contains a subunit indistinguishable from that of the placental enzyme. Inhibition studies with levamisole, a compound known to distinguish liver, kidney and bone alkaline phosphatase from the intestinal isoenzyme (Van Belle, 1972), indicate that foetal intestinal, FL-cell and KB-cell alkaline phosphatase activities are inhibited to an extent that is intermediate to those demonstrated with liver and adult intestinal alkaline phosphatases. Table 2 illustrates that a concentration of levamisole of less than 1 mM is needed to achieve 50% inhibition of activity of liver alkaline phosphatase (I_{50}), whereas the I_{50} for adult intestinal alkaline phosphatase is 5 mM and is approx. 2 mM for

Table 1. Comparison of isoelectric point (pI) and migration relative to dye front (R_F) of various alkaline phosphatases as determined from two-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

For experimental details see the text. Apparent molecular weights (M_r) were interpolated from R_F values of the following standard proteins: placental alkaline phosphatase (M_r 64 000); bovine serum albumin (M_r 64 000); transferrin (M_r 80 000); deoxyribonuclease (M_r 165 000).

Source of enzyme	Untreated subunits		Neuraminidase-digested subunits	
	pI	M_r	pI	M_r
Foetal intestine	6.0	64 000	6.5	62 000
	5.5	72 000	5.9	70 000
Adult intestine	5.9	70 000	5.9	70 000
FL cells	6.0	64 000	6.5	62 000
	5.5	72 000	5.9	70 000
KB cells	6.0	64 000		
	5.5	72 000		
Placenta	6.0	64 000		

Table 2. Inhibition of alkaline phosphatase activities

For experimental details see the text. All points represent the means of duplicate determinations in which variation was less than 5%.

Inhibitor	Concn. (mM)	Source of enzyme	...	Inhibition of alkaline phosphatase activity (%)					
				Placenta	KB cells	FL cells	Foetal intestine	Adult intestine	Adult liver
Homoarginine	1			10.1	7.5	11.3	15.8	11.6	51.9
	10			34.2	35.9	33.8	44.7	26.3	90.4
	100			84.8	82.2	79.6	77.7	74.6	98.7
Levamisole	0.1			16.0	24.9	19.4	26.8	4.6	77.4
	1			70.3	49.5	55.4	47.7	38.5	90.0
	10			96.3	88.1	89.8	81.3	80.6	97.4
Phenylalanine	1			64.3	79.2	69.4	47.3	61.3	19.1
	10			95.9	94.7	96.0	86.5	99.1	57.6
	100			100.0	99.0	99.0	97.4	99.3	95.1
Phenylalanyl-glycylglycine	1			94.8	54.6	61.0	28.6	32.5	19.5
	10			98.9	88.1	89.4	73.3	55.5	60.4
	100			100.0	99.6	99.6	97.8	99.7	100.0

foetal intestinal, FL-cell and KB-cell alkaline phosphatases. Inhibition experiments with phenylalanyl-glycylglycine, which has been used to distinguish placental alkaline phosphatase from adult intestinal alkaline phosphatase (Mulivor *et al.*, 1978b; Benham & Harris, 1979), also show that intermediate concentrations of the tripeptide are required to suppress foetal intestinal, FL-cell and KB-cell alkaline phosphatase activity as compared with placental and adult intestinal isoenzymes. These inhibition studies are interesting in that the results are consistent with our structural data, which indicate these alkaline phosphatases have subunits common to both placental alkaline phosphatase and adult intestinal alkaline phosphatase. It should be noted that small differences were consistently found in the inhibition of foetal intestinal alkaline phosphatase compared with that of FL-cell or KB-cell alkaline phosphatase, and it is possible that this represents subtle differences in the primary structure of the subunits.

Similarly, radioimmunoassay measurements of the foetal intestinal enzyme showed it to have an intermediate-shaped dilution curve when compared with the curves obtained with the term-placental and adult intestinal enzymes (Fig. 2). The dilution curve for foetal intestinal alkaline phosphatase was similar to but not identical with the one obtained with the FL-cell or KB-cell enzyme.

Fig. 2. Radioimmunoassay of alkaline phosphatase types

The shapes of the radioimmunoassay curves generated with antiserum to placental alkaline phosphatase are compared. In each case purified ^{125}I -labelled placental alkaline phosphatase was incubated with increasing activities of crude preparations of the adult intestinal (□), foetal intestinal (■) and KB-cell (●) forms of alkaline phosphatase. These are compared with the curve generated by the purified placental form (○). Liver alkaline phosphatase showed no cross-reactivity with the anti-(placental alkaline phosphatase serum). For experimental details see the text. B/B_0 indicates the ratio of radioactivity (c.p.m.) precipitated in the presence (B) and in the absence (B_0) of unlabelled antigen.

The results obtained in the present investigation have several significant implications. The use of high-resolution two-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis has indicated that foetal intestinal alkaline phosphatase consists of two non-identical subunits, a placental subunit and a glycosylated form of the adult intestinal subunit. The data support the proposal that no new structural genes or loci are involved in the transition of foetal to adult intestinal alkaline phosphatase. It appears that the molecular change during development is accomplished by the suppression of the placental alkaline phosphatase subunit and the absence of the neuraminidase-sensitive glycosylated component from the putative adult intestinal enzyme subunit of the foetal intestinal alkaline phosphatase dimer. This change in processing has been reported to occur at between 28 and 32 weeks of gestation, as indicated by Mulivor *et al.* (1978a).

The question of whether the foetal intestinal, FL-cell and KB-cell alkaline phosphatase structure is a hetero- or homo-dimer cannot be answered conclusively by the data presented in this current study, since cross-reactivity of the different subunits could account for them being precipitated with the same antiserum. However, the observation that the acidic 72000- M_r variant subunit is not expressed alone and that it appears in quantities that are approximately equivalent (by both protein content and enzyme activity) to the more-basic 64000- M_r component corresponding to placental alkaline phosphatase suggests strongly that foetal intestinal, FL-cell and KB-cell alkaline phosphatases are heterodimers of placental alkaline phosphatase and foetal intestinal alkaline phosphatase subunits. Consistent with this explanation is the report by Singer & Herz (1978) that they observed a hybrid enzyme species that reacted with both antiserum to placental alkaline phosphatase and antiserum to adult human intestinal alkaline phosphatase and that migrated in an intermediate position during electrophoresis under non-denaturing conditions. Another, though less attractive, explanation is for an aggregate of placental alkaline phosphatase and foetal intestinal alkaline phosphatase homodimers similar to the tetrameric alkaline phosphatase of liver and placental isoenzymes observed in human first-trimester placenta by Sakiyama *et al.* (1979). Each of these explanations is consistent with the reports by Mulivor *et al.* (1978a) and Miki *et al.* (1978), who resolved only one component for foetal intestinal alkaline phosphatase in electrophoresis under non-denaturing conditions, but which did not give information about molecular weight.

It is apparent that the technique utilized in this present investigation is capable of resolving and characterizing the molecular components of the

various alkaline phosphatases and could be applied to defining more precisely the structure of the tumour isoenzymes.

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