Cytosolic aldehyde dehydrogenase (ALDH, E.C.1.2.1.3) activity is regarded as one of the principal factors determining cellular resistance to some anticancer drugs, including those of oxazaphosphorine series, i.e. cyclophosphamide and its analogs (1-3). The isoforms primarily responsible for cellular resistance are tetrameric ALDH1A1 and dimeric ALDH3A1, both inactivating a key intermediate, aldophosphamide (3, 4). It has been confirmed that resistance to cyclophosphamide may be induced in sensitive cell lines by transfer of the ALDH1A1 gene (5), while expression of antisense anti-ALDH1A1 mRNA sensitizes resistant tumor cells to this drug in vitro (6). In the recent study, Sladek et al. (7) demonstrated predictive power of ALDH activity measurements, showing significant correlation between ALDH1A1 activity in breast cancer and the outcome of cyclophosphamide therapy.

It has also been suggested that high ALDH1A1 and/or ALDH3A1 activity levels may constitute a principal reason why oxazaphosphorines are of limited or no clinical value in the treatment of vast majority of lung, gastrointestinal, salivary gland, and renal cancers, i.e., many of these cancers may be intrinsically insensitive to the oxazaphosphorines, because they express large amounts of aldehyde dehydrogenase (s) (3,4). Alternatively, several other aldehyde metabolizing enzymes, like alcohol dehydrogenase, glutathione S-transferase or diaphorase, may be involved in the inactivation of aldophosphamide (3,8). In the present study, we address the question of cytosolic ALDH isozymes activity in colon cancer, to elucidate the mechanism of apparent resistance of the latter to oxazaphosphorine chemotherapy. This project extends recently published results of Jelski et al. (9), who used another type of fluorimetric assay to determine a “total” ALDH activity in colorectal cancer samples.

Most of the published data on ALDH activity in various types of tumors are based on traditional assays, utilizing either acetaldehyde, propionic aldehyde or benzaldehyde as ALDH substrates, and measuring the rate of NADH production (8,10). These results may not be quite relevant to the oxazaphosphorine resistance
problem, since aliphatic aldehydes are metabolized mainly by mitochondrial ALDH-2 enzyme, which does not metabolize aldophosphamide in vivo (2, 3). In the present work, we employ fluorimetric, class-selective assays (11) to evaluate both ALDH1A1 and ALDH3A1 activities in colon cancer and the surrounding tissue samples, and compare the results with those found in other tissues. The fluorimetric assays, based on NAD+-dependent oxidation of two fluorogenic naphthaldehydes (Scheme 1), constitute an alternative to the classic spectrophotometric study. These assays are quite selective towards cytosolic forms of ALDH, as recently confirmed by Western-blotting analysis (12), enabling rapid evaluation of the above-mentioned activities in blood and in tissue fragments including liver and thyroid tumors (11-14). Furthermore, the assays do not depend on NADH determination, what makes them relatively insensitive to interferences by other dehydrogenase systems (11).

Both ALDH1A1 and ALDH3A1 activities detected in cancer fragments may have diagnostic and/or prognostic value for cancer therapy, e.g., estimating probability of metastases. These correlations will be subject of separate investigation (Polanowski et al., in preparation).

EXPERIMENTAL

Clinical Material
Colon tumor samples (30 cases, males-8, females-13, for the rest gender was not identified) and surrounding, apparently tumor-free colon tissue fragments (ca. 1 gram both) were obtained from surgical operations, and stored at -80°C for not more than 3 months. A subset of the samples (n=6) was analyzed without freezing, within 6 h after surgery.

The tumors were diagnosed as adenocarcinoma (15 cases), adenocarcinoma mucinosum or adenocarcinoma partim mucinosum (4 cases), adenocarcinoma tubulare exulcerans (1 case), for the remaining samples diagnoses were uncertain or unknown. Age of the patients was 50-86 years (average 70), for 8 patients the age was unknown. In Dukes’ scale of tumor advancement there were 3 samples of stage A, 8 of stage B, 3 of stage C and 4 of stage D, for the rest Dukes’ characteristics was unknown.

Sample preparation
After thawing, samples were homogenized either in sucrose solution (0.25 M), buffered with 5 mM phosphate, pH 7.5, containing EDTA (1 mM) and DTT (2 mM). The homogenates were spun at 9000 g to remove the mitochondrial fraction. The resultant S-9 fraction (cytosol and microsomes) contained typically 1-12 mg protein/mL.

Chemicals
Syntheses of 7-methoxy-1-naphthaldehyde (MONAL-71), 6-methoxy-2-naphthaldehyde (MONAL-62), and the corresponding carboxylic acids were described previously (13). Concentration of the

Scheme 1. Principle of the fluorimetric assays of ADH and ALDH isozymes.
Activities of cytosolic aldehyde dehydrogenase isozymes...

stock solutions was determined spectrophotometrically, using molar extinction coefficients of 6100 and 14200 for MONAL 71 and MONAL 62, respectively, and 2100/7100 for the corresponding carboxylates (14). NAD+, NADPH+ and dithiothreitol (DTT) were purchased from SIGMA (St. Louis, U.S.A.) and all other chemicals were of analytical grade. All assays were run using disposable plastic cuvettes (Cole Parmer Corp., U.S.A.).

**Apparatus**

Fluorimetric assays were run on a thermostated Shimadzu 5001PC instrument. Instrumental settings were as follows: excitation wavelengths, 310 nm for MONAL-62, 330 nm for MONAL-71, emission was monitored at 360 nm for the oxidation product of MONAL-62, and at 390 nm for the carboxylate produced from MONAL-71. Spectral bandwidths were 3 nm for excitation and 10-15 nm for emission beams. All spectrophotometric measurements were done on a UV – 1601 PC Shimadzu instrument.

**Fluorimetric Procedure**

The activities of ALDH1A1 and ALDH3A1, were determined according to previously published procedures (11-14). The principle of these procedures is shown on Scheme 1. All these assays utilize highly fluorogenic naphthaldehyde substrates (see Scheme 1). In particular, 7-methoxy-1-naphthaldehyde (MONAL-71) and 6-methoxy-2-naphthaldehyde (MONAL-62), acting with NAD+ and NADP+ as co-substrates, respectively, are used to measure selectively the activities of ALDH1A1 and ALDH3A1 isozymes.

Fluorimetric assays were run in 50 mM pyrophosphate/HCl buffer pH 8.1, at 25°C, in the presence of 2 mM DTT and 0.5 mM EDTA. The homogenates were diluted ca. 15-fold, resulting in final protein concentration in the cuvette < 1 mg/mL. Typical substrate concentrations were 4-5 mM, justified by sub-micromolar Km values for the naphthaldehyde substrates (cf. refs. 11, 13). Coenzymes NAD+ and NADP+ (Sigma) were used in concentrations of 100 and 300 µM to measure ALDH1A1 and ALDH3A1 activities, respectively. Fluorescence background drift, if any, was measured prior to coenzyme addition and subtracted from the final slope. This background, which is due to various forms of aldehyde oxidase(s), never exceeded 10% of the final reaction rate, and in many cases was undetectable.

Purified reaction product(s) at concentrations of 3-5 µM were used as internal standards to obtain absolute reaction rates, which were calculated according to the formula:

\[ v = \frac{dF}{dt} \frac{C_s}{f_s} \]

where dF/dt is fluorescence slope, C_s, standard concentration, F_s, fluorescence of the standard.

<table>
<thead>
<tr>
<th>Isozyme/localization</th>
<th>Average specific activity [U/g]</th>
<th>C.V. [%]</th>
<th>Range</th>
<th>Number of inactive samples</th>
<th>correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH1A1 – tumor</td>
<td>0.081</td>
<td>133</td>
<td>0–0.47</td>
<td>5</td>
<td>0.12</td>
</tr>
<tr>
<td>– tumor-free tissue</td>
<td>0.096</td>
<td>87</td>
<td>0–0.33</td>
<td>2</td>
<td>-0.01</td>
</tr>
<tr>
<td>ALDH3A1 – tumor</td>
<td>0.065</td>
<td>121</td>
<td>0–0.38</td>
<td>2</td>
<td>-0.01</td>
</tr>
<tr>
<td>– tumor-free tissue</td>
<td>0.093</td>
<td>129</td>
<td>0–0.63</td>
<td>2</td>
<td>-0.01</td>
</tr>
<tr>
<td>ALDH1A1 – males* tumor</td>
<td>0.056</td>
<td>101</td>
<td>0–0.13</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>– tumor-free tissue</td>
<td>0.096</td>
<td>123</td>
<td>0–0.33</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>ALDH1A1 – females* tumor</td>
<td>0.085</td>
<td>143</td>
<td>0–0.47</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>– tumor-free tissue</td>
<td>0.074</td>
<td>76</td>
<td>0–0.21</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>ALDH3A1 – males- tumor</td>
<td>0.041*</td>
<td>78</td>
<td>0.01–0.11</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>– tumor-free tissue</td>
<td>0.069</td>
<td>140</td>
<td>0–0.30</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>ALDH3A1 – females tumor</td>
<td>0.109*</td>
<td>95</td>
<td>0–0.38</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>– tumor-free tissue</td>
<td>0.130</td>
<td>122</td>
<td>0.01–0.63</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

* limit of sensitivity is ~0.01 U/g for each isozyme.

N = 13 for female and 8 for male samples.
Sensitivity limits for reaction rates were typically ~0.1 nM/min, which corresponded to specific activities of ~0.01 U/gram protein (14). Enzyme unit is defined as an amount of enzyme catalyzing oxidation/reduction of one micromole of the naphthaldehyde substrate per minute. As indicated in ref. 11, the naphthaldehyde activities are close, but not identical, to those based on acetaldehyde/benzaldehyde oxidation.

Protein concentration in tissue homogenates was determined (in mg/mL) by means of the Bradford method, using Sigma kit and BSA as standard references.

RESULTS AND DISCUSSION

Cytosolic forms of aldehyde dehydrogenase, ALDH1A1 and ALDH3A1, are two main ALDH forms responsible for \textit{in vivo} oxidation of aldo-phosphamide, a key intermediate in cyclophosphamide biotransformation (2,3). ALDH1A1 is expressed in nearly all human tissues examined to date.

Figure 1. Activities of ALDH1A1 (panel A) and ALDH3A1 (panel B) in colon tumors (open bars) and the surrounding tissue fragment homogenates (filled bars), as determined by the fluorimetric method. Assays were run in 20 mM pyrophosphate buffer, pH 8.1, at 25°C.
although with significant quantitative differences (1-3,7,11-14). By contrast, ALDH3A1 is recognized as an inducible enzyme in liver and some other organs (2), but constitutively present in saliva, lungs and stomach (2, 3), and most of all, in cornea (15), where it presumably plays protective role against UV-induced tissue damage. Selective measurement of these two activities in tumors have significant predictive potential for oxazaphosphorine chemo-therapy of breast cancer (7).

In the present study, we have measured the activities of both isozymes using fluorimetric assay, based on oxidation of fluorogenic naphthaldehydes (11-14). In contrast to the standard ALDH assays, based on acetaldehyde or propionic aldehyde oxidation, the fluorimetric assay is virtually insensitive to the mitochondrial form of the enzyme, ALDH2A1 (11), which does not participate in cyclophosphamide inactivation process (2). Further proof of its high selectivity comes from kinetic analysis (11,13,14), competition with non-fluorescent substrates (13-14), and from comparison of the results obtained by fluorimetric method vs. Western blot analysis (12), showing semi-quantitative agreement between these two methods, especially well pronounced for ALDH1A1. Importantly, both naphthaldehydes do not produce NAD(P)+-dependent fluorescence signal in the presence of diluted healthy human plasma (11,16), and MONAL-71 is, additionally, not oxidized by human saliva (13), indicating resistance to all aldehyde-oxidizing enzymes that are present in these media.

Using fluorimetric assays described above, we have found that both ALDH activities are measurable in most of 30 colon tumor sample homogenates examined, as well as in the surrounding tissue fragments. Both tumor and tissue activities are highly variable, and the pathological fragments may differ several-fold in activity from the tumor-free tissue. The results are summarized in Table 1.

The ALDH1A1 activity, as determined with MONAL-71/NAD+ assay, in most colon tissue fragments is close to 0.1 U/g, which is more than 1 order of magnitude lower than that found in the liver (cf. ref. 14), and comparable to the activities found in thyroid fragments (14). This activity is in many cases still lowered in cancerous tissue, although average values do not differ significantly (Table 1). There were 5 cases of non-detectable (< 0.01 U/g) activity in tumors and 2 inactive samples of colon tissue. We found at least 2 cases of measurable ALDH1A1 activity found in the surrounding tissue, but not in the tumor. The MONAL-71 activities can be directly compared to acetaldehyde (200 µM) or propionic aldehyde (100 µM) activities, due to similar oxidation rates found for these substrates (cf. Table 1 and refs. 11,13).

The apparent activities of ALDH3A1 in cancer-free colon tissue, as determined by the fluorimetric method, (0 – 0.6 U/g, cf. Table 1) are generally much lower than those found in stomach (2-5 U/g) or in livers of cancer patients (cf. refs. 13,14). These activities are highly variable both in tumors and in cancer-free tissue (Table 1), but average values are not significantly different. Some tumor and tumor-free samples did not contain any detectable ALDH3A1 activities (2 cases each, with several other samples on the verge of detectability).

There was no significant difference in ALDH1A1 activity between male and female patients, neither in tissue nor in tumors. Surprisingly, the apparent ALDH3A1 activity was higher in female group, especially regarding the tumor activity (Table 1). This result must be treated with some caution due to limited number of cases.

In agreement with the literature data, we did not find any correlation between ALDH activity and patient’s age in colon tissue (r = – 0.02 and – 0.23 for ALDH1A1 and ALDH3A1 activities, respectively). In tumors, the respective values were 0.08 and 0.36. There was also no correlation between tumor and tissue activity (Table 1). Two measured ALDH activities in tumors were also poorly correlated (r = 0.25).

There is no direct relation between the Dukes’ characteristics of tumor advancement and ALDH activities in tumors (data not shown), although statistics is in this instance not sufficient to formulate final conclusions.

To determine an influence of sample storage conditions on ALDH activity in clinical material, sample #15 has been examined directly after the operation (~6 h delay), and part of the material has been frozen and re-examined again after ~30 days. The activities of two forms of ALDH were lowered by ~15%, showing limited influence of storage conditions on enzymatic activities. Five other samples were examined without freezing, within 6 h after surgical operations. The resultant average activities for ALDH1A1 were 0.070 U/g and 0.060 U/g in tissue and in tumor, respectively, and the analogous values for ALDH3A1 were 0.099 and 0.079. These values do not differ significantly from general data presented in Table 1.

In an additional test, two samples selected on the basis of their activity and mass, were divided in two parts, one of which was examined after less than
3 months, and another after 1 year of storage at −80°C, with resultant loss in activity for ALDH1A1 and ALDH3A1 less than 20%. Although this result may be somewhat distorted due to inhomogeneity of the clinical material, it shows that even prolonged freezing does not cause inactivation of the ALDH activity. In a separate experiment, apparently homogeneous liver samples were subjected to consecutive freezing-and-thawing cycles, resulting in 20-35% decrease in ALDH activity for each cycle (data not shown). It seems therefore that it is rather the freezing/defreezing procedure that affects ALDH activity, than the storage itself. In the present paper, only results of examination of once-frozen samples, stored for no longer than 3 months are reported.

The results presented here differ from those reported by Hengstler et al. (8), and others (10), which suggested high and rather constant ALDH activities in colon tumors, with little or no differences between tumor and healthy tissue activity. By contrast, Yin et al. (18), using isoelectric focusing, demonstrated much lower ALDH1A1 activities in tumors in comparison with the healthy colon tissue. These apparent discrepancies may be due to the different method of enzyme assays, i.e., the traditional method employed by Hengstler et al. is sensitive primarily to the mitochondrial ALDH-2, an isozyme constitutively present in all cells except erythrocytes (2, 3), or to inherent difficulties of the acetaldehyde method (13).

We have found average specific activities of two ALDH isozymes in colon of the order of 0.1 U/g, which is 10-50 fold less than the corresponding values found in liver and stomach homogenates, but comparable to thyroid ALDH1A1 activity, evaluated by the same method (13, 14). Our results are also in qualitative agreement with those published recently by Jelski et al. (9), and referred to as “total” ALDH. Since thyroid tumors are frequently sensitive to oxazaphosphorines (17), we conclude that ALDH activity alone (either in ALDH1A1 or in ALDH3A1 form) is probably not responsible for the reported resistance of the colon tumors to this class of drugs. By contrast, elevated level of class I alcohol dehydrogenase in cancerous tissue, reported recently by Jelski et al. (9), and confirmed by us (data not shown), may be important for the oxazaphosphorine metabolism.

Although ALDH1A1 is not an inducible enzyme, significant variability of its activity was observed in the apparently healthy colon tissue (Table 1). This somewhat surprising fact can be explained on the basis of the well-known sensitivity of ALDH to oxidation (19). It is known that blood ALDH, which consists mostly of ALDH1A1 isozyme, undergoes inactivation by many drugs (e.g., nitrates), and is also sensitive to patients’ drinking and smoking habits, presumably via the enzyme oxidation mechanism (16, 19). Analogous mechanism may be also responsible for colon ALDH1A1 variability (cf. ref. 20). Recent investigations on other tumors, e.g. breast cancer, utilizing ELISA, revealed similarly large variability of both ALDH1A1 and ALDH3A1 content (21).

Acknowledgements

We express our gratitude to Professor Krzysztof Bielecki (Warsaw Medical School) for providing some of the surgical samples, and to Dr. N.E. Sladék for his critical comments. This paper was supported by Medical School, Warsaw.

REFERENCES


Received: 20.08.2005