Association of Levels of Circulating Clq Binding Macromolecules with Induction Chemotherapy Response in Head and Neck Cancer Patients

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ABSTRACT

Ninety-five untreated patients with squamous cell carcinoma of the upper aerodigestive tract expressed significantly higher levels of Clq-binding macromolecules as compared to 45 noncancer-bearing controls. No relationship between Clq-binding macromolecules and levels of circulating IgG-immune complexes as determined by the solid-phase Clq-binding assay or the C3d-solid-phase assay could be defined suggesting that Clq-binding macromolecules were distinct from IgG-circulating immune complexes. An elevated level of Clq-binding macromolecules within these patients was predictive of subsequent response to induction chemotherapy; those with elevated levels characteristically showed no response. Using multivariate logistic regression analysis including the covariates of American Joint Committee staging parameters as well as Clq assay results, levels of the isolated macromolecules added significant prognostic information to the probability of chemotherapeutic response. The quantitation of Clq macromolecules has clinical implications as to choice of therapeutic regimens against head and neck cancer. The nature of these substances remains to be defined.

INTRODUCTION

Recent therapeutic approaches against head and neck cancer involve attempts to both control tumor progression and yet preserve critical patients functional capacities including speech and/or deglutition. Foremost of these recent approaches has been the use of induction chemotherapy (1). Multidrug regimens consisting principally of cisplatin, bleomycin, and/or 5-FU have generated antitumor response rates ranging from 48 to 84% when applied prior to surgery and radiation therapy (2-6). Complete tumor regression following the use of such therapeutic agents included i.v. combination cisplatin, bleomycin, and 5-fluorouracil as previously described (5). The agent cis-platinum was given at a dose of 100 mg/m2 on Day 1. Bleomycin was given by continuous infusion on Days 1-3 at a dose of 15 U/m2. 5-FU was likewise given by continuous infusion; 1000 mg/m2, Days 1-4. Each cycle was repeated at 21-day intervals for a total of three cycles. If no response was noted after the second cycle, chemotherapy was terminated and patient subsequently underwent surgical resection of residual cancer. Patients were considered eligible for chemotherapy if they had Stage III or Stage IV disease, no evidence of distant metastases, and an adequate performance status (Karnofsky performance status greater than 60%). The male to female ratio was 2.9 to 1. Disease stage was determined according to AJC criteria (10) and was assigned by members of the Department of Head and Neck Surgery without knowledge of Clq binding values. The primary diagnoses included 24 patients with oral cavity lesions, 42 patients with pharyngeal cancers, and 27 patients with laryngeal cancers. Two patients had multiple primary cancers, a situation that precluded defining one particular disease site. Treatment consisted of single- or combined-modality therapy, primarily surgery or radiation or both. Twenty-nine of the 95 patients underwent induction chemotherapy as their initial treatment. Chemotherapeutic agents included i.v. combination cis-platin, bleomycin, and 5-fluorouracil as previously described (5). The agent cis-platinum was given at a dose of 100 mg/m2 on Day 1. Bleomycin was given by continuous infusion on Days 1-3 at a dose of 15 U/m2. 5-FU was likewise given by continuous infusion; 1000 mg/m2, Days 1-4. Each cycle was repeated at 21-day intervals for a total of three cycles. If no response was noted after the second cycle, chemotherapy was terminated and patient subsequently underwent surgical resection of residual cancer. Patients were considered eligible for chemotherapy if they had Stage III or Stage IV disease, no evidence of distant metastases, and an adequate performance status (Karnofsky performance status greater than 80%). Specific hematological, hepatic, renal, and pulmonary status had to be met (5). Chemotherapy was chosen in such cases because a response to chemotherapy would preclude surgery that would impair speech and/or deglutition. Response to chemotherapy was determined initially by members of the Division of Medicine (Section of Head and Neck Medical Oncology) and corroborated by independent examiners from the Department of Head and Neck Surgery. No response to treatment was defined as less than a 50% reduction in tumor bulk at the primary site. A partial response was defined as more than a 50% reduction but with clinically apparent tumor still remaining. Finally, a response was considered complete if no clinically evident disease was noted at the primary site after completion of chemotherapy. All examiners were blinded to the patients' results of Clq-binding assay. Likewise, laboratory results were tabulated prior to therapy without knowledge of disease status or subsequent treatment response. The control population consisted of 45 randomly chosen normal individuals who were free of active disease and were without previous history of cancer. The controls were chosen so as to be equivalent in age to the cancer patients. The median age of the controls was 59.5 years.
years (range, 36–76 years); age not significantly different from the cancer group (P = 0.81). The male to female ratio was 0.89 to 1.

Measurement of CIC

Three methods were used to measure immune complexes.

Clq-binding Test. Sera were collected from clotted blood and then frozen at –70°C until tested (11). Clq, purified by the method of Kolb et al. (12), was labeled with 125I by the iodobead method (Pierce Chemical Co., Rockford, IL). Briefly, 500 μg of Clq were mixed with 1 mCi of sodium 125I in 100 μl of PBS, pH 7.4, in a final volume of 1 ml on ice. One iodobead was added to this mixture and incubation was continued for 15 min. Labeled Clq was separated from free 125I on a 10-cm column of Sephadex G-25 (PD-10 column, Pharmacia, Piscataway, NJ). The Clq binding test was performed as previously described, using EDTA-treated sera according to the method of Zubirei et al. (11, 13). Results were expressed as μg/ml equivalents of heat aggregated IgG, with reference to a standard curve made with purified IgG, aggregated at 63°C for 30 minutes at a concentration of 3 mg/ml and diluted serially in heat inactivated (56°C, 30 min) normal donor serum. Sera from healthy, age-matched normal donors were used as negative controls and sera from patients with rheumatoid arthritis provided positive controls in each test run.

Solid-phase Assays for Circulating Immune Complexes Bearing C3d and Clq. For these assays we used microtiter plates coated with murine monoclonal antibodies specific for either human Clq or human C3d (Immunomedics, Inc., Newark, NJ). Sera, stored at –70°C, were thawed and diluted 1:5 in a proprietary diluent (PBS containing heat-inactivated horse serum and polyoxyethylene sorbitan monolaurate). Diluted sera, diluent controls, and serially diluted standards containing heat-aggregated IgG, were added to the microtiter plate wells in 200-μl aliquots. After incubation at 32 to 37°C for 60 min, the microtiter plates were emptied and washed with the PBS-polyoxyethylene sorbitan monolaurate three times; then horseradish peroxidase-conjugated monoclonal antibody to human IgG was added to the wells. Incubation was continued at room temperature in the dark, and o-phenylenediamine and hydrogen peroxide were added to develop color. Absorbance at 488–492 nm was read on a Dynatech MR 580 microelisa reader; the color developed by the test specimens was related to that resulting from serial dilutions of heat-aggregated IgG in the standards, using a least-squares linear regression program available for the Hewlett-Packard model 97 calculator.

Statistics

Differences between two populations regarding an indicated laboratory parameter were tested for significance using the Mann-Whitney U test. The correlation between two laboratory parameters was analyzed by means of Pearson's correlational analysis. All other statistical assessments are indicated where appropriate within the text. P-values less than 0.05 were considered significant.

RESULTS

Disease Stage and Levels of Clq Binding Macromolecules. The distribution in levels of binding macromolecules determined by the Clq binding test in 95 head and neck cancer patients and 45 healthy age-matched controls is shown in Fig. 1. The overall levels of Clq binding macromolecules were significantly higher in the cancer population than in controls (49.6 ± 48 μg/ml versus 20.1 ± 23 μg/ml) (P < 0.001).

Considering that no single test for immune complexes is comprehensive (14) and because of the likelihood that the Clq-binding method may detect macromolecules in addition to immune complexes (15–25), we evaluated a subset of these patients’ sera for immune complexes by two solid-phase ELISAs designed to detect immunoglobulin associated with circulating Clq or C3d. The choice of the 43 patients’ sera was dictated solely by the availability of sufficient quantities of sera that a fresh aliquot could be thawed for each of the assays. No difference between head and neck cancer patients and controls could be identified using the solid-phase C3d and Clq ELISAs (Fig. 2). Only the Clq-binding tests effectively discriminated the patients from the healthy controls (35.3 ± 40.9 μg/ml versus 15.6 ± 15.4 μg/ml) (P = 0.01). Regression analyses showed that values obtained from the two solid-phase assays correlated (r = 0.37) (P < 0.01), but values from the liquid-phase Clq assay did not correlate with either solid-phase assay. Considering these results, analyses of the clinical significance of measurements of these macromolecules were restricted to the results obtained with the Clq-binding test.

The relationship of levels of Clq-binding macromolecules to tumor stage and site of primary cancer as defined by AJC staging parameters is shown in Fig. 3 and Table 1. High levels of Clq-binding substances in patients with advanced stage disease was a function of the size of the primary tumor: Patients with T3 T4 primaries (N = 56) had a mean CIC level of 57.4 ± 51 μg/ml as compared to a mean of 39.8 ± 43 μg/ml noted in patients with T1 T2 primaries (N = 37) (P < 0.05). T-stage could not be assigned in two patients because previous diagnostic surgical biopsies prior to referral for definitive therapy precluded accurate assessment. No significant differences in levels of Clq-binding macromolecules were identified between patients with or without clinical evidence of regional nodal metastases (53.6 ± 48 μg/ml versus 46.0 ± 49 μg/ml, respectively) (P = 0.31). Likewise, no significant differences could be
Chemotherapy as their initial treatment. The remaining individ-
uals were treated as follows: surgery alone (12 patients); radiation
therapy alone (17 patients); combination surgery and radi-
ation therapy (20 patients). Seventeen patients had either
partial or complete response to chemotherapy was significantly
lower than that of the 15 patients with no tumor response (23.1
± 30 µg/ml versus 85.7 ± 54 µg/ml, respectively) (P < 0.005).

We assessed whether a primary tumor's failure to respond to
chemotherapy depended on C1q binding levels. Patients were
stratified by both stage of disease and by C1q binding values
(Fig. 4). A significant relationship was noted between tumor
response and pretreatment level of C1q binding macromole-
cules. The mean values of the 14 patients who had either a
partial or complete response to chemotherapy was significantly
lower than that of the 15 patients with no tumor response (23.1
± 30 µg/ml versus 85.7 ± 54 µg/ml, respectively) (P < 0.005).

To examine whether measurement of C1q-binding substances
improves our ability to predict response to treatment, a logistic
regression analysis was performed using stage, site of disease,
and C1q binding test results and was compared to a model
without C1q-binding measurements. Measurement of C1q-
binding substances improved the fit of the model significantly
(χ² = 12.4, 1 df, P < 0.001). A further comparison with a
statistical model incorporating only C1q-binding substances
shows that the stage and site add predictive ability to the C1q
values (χ² = 9.15, 2 df, P = 0.01). Thus, measurement of C1q-
binding substances together with AJC staging parameters are
more predictive of response to induction chemotherapy when
considered together than when considered independently.

In order to judge the size of the prognostic effect, quantiles
of C1q-binding substances were calculated separately for each
site and stage combination, and the estimated response at that
quantile obtained from the regression equation. Quantiles are
C1q-binding values such that a specified proportion of the site-
stage population had lower values. Thus, 25% of the Stage IV
pharynx values are less than or equal to the 0.25 quantile. The
results of this calculation are plotted in Fig. 5.

The estimated size of the prognostic effect is striking. For all
site and stage combinations, the estimated response rate for
not a factor in chemotherapy response in this group of individ-
uals. (χ² = 0.28, 1 df) (P < 0.75). Analysis (χ²) revealed that
Stage III cancer patients were more likely to respond to chem-
otherapy than Stage IV patients. This difference did not, how-
ever, reach a statistically significant level (χ² = 2.77, 1 df) (P
< 0.10).

Table 1

<table>
<thead>
<tr>
<th>Site</th>
<th>Stage I and II</th>
<th>Stage III and IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>38.9 ± 43 (8)</td>
<td>52.6 ± 36 (15)</td>
<td>47.9 ± 38 (23)</td>
</tr>
<tr>
<td>Pharynx</td>
<td>53.1 ± 67 (7)</td>
<td>54.9 ± 50 (34)</td>
<td>54.6 ± 52 (41)</td>
</tr>
<tr>
<td>Larynx</td>
<td>39.8 ± 38 (10)</td>
<td>46.1 ± 58 (17)</td>
<td>43.8 ± 50 (27)</td>
</tr>
<tr>
<td>Total</td>
<td>43.2 ± 47 (25)</td>
<td>52.1 ± 49 (66)</td>
<td>49.7 ± 48 (91)</td>
</tr>
</tbody>
</table>

* C1q binding levels were determined using serum samples previously frozen
at −70°C and are expressed as µg/ml equivalents of heat aggregated IgG. All
values are expressed as the mean ± 1 SD.
* Site and stage of disease as determined by AJC staging system.
* Four of the total 95 evaluable patients were excluded from this analysis: Two
had multiple primary cancers and two patients had previous diagnostic biopsies
which precluded specific site and stage classification.
* Numbers in parentheses, numbers of patients.
* P < 0.005 as compared with 45 noncancer bearing controls with mean binding
levels of 20.1 ± 23 µg/ml.
* P < 0.05 as compared with 45 noncancer bearing controls.

identified when comparing patients with combined Stage I and
Stage II disease versus combined Stage III and Stage IV patients
(P = 0.31) (Table 1).

C1q Binding Macromolecules and Response to Induction
Chemotherapy. Twenty-nine individuals underwent induction
chemotherapy as their initial treatment. The remaining individ-
uals were treated as follows: surgery alone (12 patients); radiation
therapy alone (17 patients); combination surgery and radio-
diation therapy (20 patients). Seventeen patients had either
been treated elsewhere or had no completed primary therapy at
the time of this assessment.

In the 49 patients who were treated at our institution by
means other than induction chemotherapy, no relationship
between the level of C1q-binding substances and response to
treatment could be identified.

Of the 29 individuals who underwent induction chemother-
apy, 12 patients had Stage III and 17 had Stage IV disease.
Seven had laryngeal cancer, 18 had pharyngeal cancer, three
had oral cavity primaries, and one patient had multiple primary
cancers.

Response to chemotherapy was analyzed as a function of site
and stage of disease. Because of the few individuals with oral
cavity cancer, individuals with larynx and oral cavity cancer
were combined for comparison with the pharynx cancer popu-
lation. Likewise, because of the small number of patients and
the need for parsimony in chemotherapy response analysis,
patients were categorized as either chemotherapy responders
(partial or complete) versus nonresponders. Site of disease was

Fig. 3. Individual C1q-binding levels in patients with head and neck cancer
categorized by site and stage of disease. Disease staging was by AJC methods
(10). © 1988 American Association for Cancer Research. Cancer Res. 52: 5862-
5869, 1992. Fig. 4. The relationship between stage of disease, levels of C1q-binding
macromolecules, and response to induction chemotherapy. Head and neck cancer
staging was by AJC methods (10). © 1988 American Association for Cancer Research.
It is notably difficult to predict head and neck cancer patient's response to induction chemotherapy. Tumor regression rates range from 48 to 84% depending on drugs used and patient status (2–6, 28, 29). Tumor factors considered relevant to response to treatment include cellular differentiation, disease site, and extent of tumor burden (3, 4, 6, 28–30). However, no unanimity exists as to the relative importance of each of these factors. Our results agree with those who assign significance to tumor stage (3, 4, 6, 28, 29). Patients with Stage IV head and neck cancer responded less well than individuals with Stage III disease. The overall response rate (48%) in this study, however, is lower than previous studies utilizing combinations of agents consisting principally of cis-platinum and 5-FU. The relationship of disease stage to treatment response and the lack of any chemotherapy patients with early stage (Stage I and II) disease in this study may account for such differences. Despite our overall low response rates, the significance of our results lies in the additional prognostic values acquired by measuring Clq-binding substances in conjunction with standard staging techniques in evaluating patients for chemotherapy. Logistic regression analysis including the covariates of site of disease, stage, and levels of Clq-binding macromolecules demonstrated that the measurement of Clq-binding substances significantly improves the model. Thus, the in vitro measurement of these substances adds to our ability to predict head and neck cancer patients' response to chemotherapy beyond that provided by standard clinical assessment.

It is also noteworthy that these studies have demonstrated the presence of immune complex-like substances in the sera of patients by means of the Clq-binding test but not by assays which are specifically designed to detect immunoglobulin G bound in macromolecular complexes with either Clq or C3. It is conceivable that the “immune complexes” in these sera exclusively incorporated antibodies of the IgM or IgA isotypes, and therefore cannot be detected by the ELISA tests used in these studies. Alternatively, and more likely is the possibility that the Clq-binding substances in these sera may not have been immune complexes at all. Cellular and subcellular membranes from heart, liver, and brain from humans and other mammals bind human Clq with high affinity ($K_a = 10^{7}$ to $10^{10}$ M$^{-1}$) (15). These membranes also activate Clq, as demonstrated by the conversion of $^{125}$I-labeled Clq to activated Clq. There are a large number of substances which can bind Clq and activate Clq, in addition to complexed immunoglobulin, or immunoglobulin aggregates (16–22). These include the lipid A region of lipopolysaccharides (16), certain RNA tumor viruses (17), complexes containing C-reactive protein (19), and mitochondrial membranes, most notably of cardiac origin (22). Indeed, recent studies by Rossen et al. (23) have shown that within the first 72 h following a coronary occlusion, patients with documented myocardial infarctions frequently have elevated levels of serum Clq-binding activity. This is seen in patients who have no evidence of antibody to myocardial tissue antigens; experimental studies suggest that the Clq-binding substances in these circumstances are subunits of cardiac mitochondria which are released into the extracellular fluids surrounding damaged myocardial cells which reach the plasma via the lymphatics (23, 24). It has been suggested that the integral membrane component in mitochondria responsible for binding and activating Clq is cardiolipin (25). Thus, patients with head and neck tumors who have abnormally high levels of serum Clq-binding activity may have circulating subcellular organelles, possibly of tumor cell origin, which fix Clq. Investigations are presently underway to address this question.

**DISCUSSION**

Head and neck cancer patients can be characterized as having elevated serum levels of Clq-binding macromolecules as compared to a noncancer-bearing control population. Should these substances prove to be circulating immune complexes, then results would be consistent with previous investigations which document elevated immune complex levels in head and neck cancer patients (26, 27). The clinical relevance of such measurements relates to subsequent response to induction chemotherapy. Those individuals who failed to respond to chemotherapy had significantly higher levels of Clq-binding substances than did those who responded to chemotherapy. Results extend observations by Denaro et al. regarding the prognostic implication of measuring immune complex-like substances within sera of head and neck cancer patients (27). Using a PEG precipitation technique, Denaro et al. noted that levels of precipitable protein correlated with the tumor-bearing state (27). With surgical removal of tumor, levels of PEG-precipitable protein diminished except in those patients who subsequently developed disease recurrence. Thus, PEG-precipitate levels in the early posttreatment period appear to be a marker associated with a high probability of relapse (27).
Not provided in this investigation, nor yet available, is evidence which explains why patients with a elevated Clq-binding macromolecules fail to respond to chemotherapy. One could first hypothesize the relationship simply as an associated phenomenon. Clq-binding macromolecules may by themselves play no direct role. The presence of the substance may reflect enhanced tumor kinetics, i.e., enhanced cellular division, a shortened cell cycle, and associated increase in tumor cell death. As tumor cells die, just as occurs with myocardial necrosis (22, 23), cancer-related macromolecules capable of binding complement may be shed into peripheral blood. The antitumor effect of chemotherapy utilized in this setting may simply fail to "keep pace" with tumor growth. Overall tumor mass may remain unaffected or even increase during therapy. Historically, the rapidly progressive disease leading to shortened survival in those patients who demonstrate no response to chemotherapy would support this hypothesis (3, 4). Additionally, the more rapidly proliferating cells in this setting may have enhanced DNA repair systems thereby making them more resistant to DNA damage by such agents as cis-platinum (31–33).

Alternatively, Clq-binding macromolecules may play a more direct role. One mechanism may involve macromolecule binding of chemotherapeutic agents within peripheral blood causing a dilutional effect, i.e., less drug would be made available to bind tumor. Cis-platinum has been repeatedly shown capable of binding protein within peripheral blood with resultant alterations in drug clearance and diminished cytotoxic capacity (34–36). An additional explanation relates to investigations by Shearer and Terman (37–39). The explanation may be valid should future investigations demonstrate that complement-binding substances within the head and neck cancer patient are antigen-antibody complexes. Shearer has noted that tumor-associated antibodies will induce an increased uptake of cyto- sine arabinoside upon binding with the target cell (37). Increased tumor cell death subsequently occurred (37). Terman noted that with removal of tumor antigen-antibody complexes through plasmapheresis, free antibody deposition is increased within the primary tumor site (38). Sequential infusion of cytosine arabinoside following plasmapheresis induced a synergistic antitumor effect in Terman’s in vivo animal breast cancer model (39). Antibody which is not complexed with circulating tumor proteins may be more readily available to potentiate drug uptake through mechanisms of increased drug phosphorylation mediated by antibody bound to tumor cells (40). Stimulated by the clinical significance of measuring Clq-binding macromolecules, one should be able to elucidate the mechanisms which account for these observations.

REFERENCES

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