Technical Advance

Improved Analysis of the Vascular Response to Arterial Ligation Using a Multivariate Approach

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Ligation of the murine common carotid artery induces a reproducible remodeling response. The contribution of individual genes can be determined by comparison of the phenotypes of genetically modified mice. Although studies have shown the response to carotid artery ligation is influenced by many factors, individual analyses typically only consider a single factor, the presence of a gene of interest. Because of this limitation, measurements of the response to ligation show large variation, making the determination of significant difference between test groups difficult. In this study, we examine the hypothesis that the variation in the response to ligation is due to non-genetic factors in addition to genetic factors. Distance from the ligature, a variable common to all arterial ligation experiments, is an important source of variation and a significant predictor of the remodeling response. We find that the use of statistical regression is an improved analysis technique, as it allows the simultaneous consideration of multiple variables. We demonstrate this by showing improved sensitivity and novel findings in the analysis of the remodeling response in mice genetically mutant for the osteopontin gene. We conclude regression analysis provides a simple way to improve both comparative power and description of vascular remodeling. (*Am J Pathol* 2004, 164:43–48)

Chronic obstructive vascular pathology (eg, stenosis, restenosis, arteriosclerosis) remains a widespread health problem and the largest contributor to the morbidity and mortality of both men and women in developed nations.¹ These pathologies each involve a response to a physical stimulus, such as changes in shear stress, direct injury, or oxidative injury, resulting in vascular remodeling and neointima formation. As obstructive pathologies have been shown to involve complex regulation of several cell types, there continues to be great need for *in vivo* models to understand the mechanistic basis of the pathology.

Murine models of vascular disease are especially useful in determining the contribution of specific gene products as genetically modified mice are easily generated and readily available. Current models include mice that spontaneously demonstrate chronic obstructive vascular disease² and induction of obstruction by direct injury of the vasculature.^{3–7} The complete ligation of the carotid artery is one method of inducing a vascular remodeling response in mice. Originally described by Kumar and Lindner,⁷ this model has the advantages of being technically simple to perform and yielding a highly reproducible localized vascular remodeling response. This murine model is being increasingly used, and has been instrumental in defining the contributions of plasma protein systems,^{3,8,9} cytokines,^{10,11} and physical forces such as pressure¹² and shear stress⁷ in the vascular remodeling response.

Whereas the vascular lesions formed in response to some injuries such as endothelial denudation are relatively consistent over the involved arterial segment, carotid artery ligation induces a response that is generally greatest at the ligature, and gradually diminishes over the involved arterial segment (3 to 5 mm from the site of ligation). This variation with distance from the ligature was noted in the original paper describing murine carotid artery ligation,⁷ and the need to consider the effect of distance in analysis was recognized by Yogo et al¹³ and Sindermann et al.¹⁴ As the occasional occurrence of thrombus, typically close to the ligature, is another source of variation in this model, most studies have discarded the 1 mm segment closest to the ligature from the analyzed data set. In previous studies using this model, data analysis has occurred in one of two ways: determination of mean response over an entire arterial segment, or limitation of analysis to a specific portion of the segment. Both of these methods of analysis have problems that limit interpretation. In the first method, taking a mean value over a length of vessel that is changing in a predictable manner masks differences in particular regions

Supported by a grant from the American Heart Association to L.L. (025250N).

Accepted for publication September 10, 2003.

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between groups, and results in an especially large standard deviation. On the other hand, limiting analysis to comparable arterial regions has difficulties as the method uses a smaller data set and inspection of data before analysis violates statistical premises. In this study, we examine the hypothesis that much of the variation in the response to ligation is due to non-genetic factors in addition to genetic factors. Our results build on previous studies that have recognized the importance of considering regional differences, with the demonstration that distance from the ligation contributes to the total variance and is a significant predictor of the remodeling response. Statistical regression is presented as means of simultaneously analyzing the contribution of several variables to the response to ligation. In essence, the method involves fitting a curve to the data set, describing the curve as a function, and then determining the variation of each data point from that function. In addition to being multivariate, this method has the advantage of portioning the total variance to each variable resulting in increased sensitivity. The practical utility of the method is then demonstrated by application to a data set testing the effects of osteopontin (OPN) on the response to carotid artery ligation.¹⁵ In addition to providing increased significance, regression analysis revealed significant regional differences due to OPN that cannot be appreciated by conventional analysis methods. As regression analysis is a commonly used statistical method, actual calculations are not presented, and the reader is referred to SAS regression procedures¹⁶ for description of the actual calculations performed.

Materials and Methods

Murine Carotid Artery Ligation Model

The data used in this study were taken from the previously published study,¹⁵ and the experimental methods are presented briefly here for clarity. All protocols were approved by the Institutional Animal Care and Use Committee. The OPN-null mutant allele has been described,¹⁷ and mice used in this study were 16- to 24-week-old males on a 129 $\times$ Black Swiss (Taconic, Germantown, NY) hybrid background. Ligation of the left carotid arteries of mice was performed as previously described⁷ on a test group of mice (wild-type, $n = 8$; OPN-null, $n = 12$) and sham operations were performed on a control group of sham-manipulated mice (wild-type, $n = 6$; OPN-null, $n = 6$). Two weeks following ligation, tissues were perfusion fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1 mol/L sodium phosphate buffer, pH 7.3. Vessels were harvested and embedded in paraffin such that initial sections from the block came from the internal/external carotid arteries. At least 800 consecutive serial sections were cut from both arteries of each mouse (7 μ m thick). Sections including the ligation were easily identified due to the clearly visible suture material. As every section was saved and numbered, the distance from the site of ligation (relative start site in the case of sham manipulation) and between sections was precisely known. Sections of the internal/external carotid arteries

were discarded. As analysis of previous work using this strain showed that substantial change in remodeling could be observed in sections 200- μ m apart, we considered sections 280- μ m apart to be independent observations. Ten sections from each mouse collected every 280- μ m apart starting at the site of ligation were analyzed, thus representing a total 3-mm segment. Sections were stained with orcein to accentuate the elastin layers, and morphometric analysis was performed. Digitized images of these vessels were analyzed using image analysis software (NIH Image 1.60). The lumen surface, the perimeter of the neointima, and the perimeter of the tunica media were traced, for circumference and area of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL), respectively. The medial area was calculated by subtracting the area defined by the IEL from the area defined by the EEL. Intimal area was calculated as the area between the lumen surface and IEL. As the shape of the cross sections is often distorted and the neointima may be unevenly distributed, lumen area could not be measured directly. Assuming the EEL was circular, lumen area was calculated by subtracting medial area and intimal area from the total area calculated from the circumference of the EEL.

Data Analysis

Data were analyzed using SAS Version 8.0. See the Table legends for descriptions of methods used for each analysis.

Results

Distance from the Site of Ligation Significantly Affects the Remodeling Response

To test the hypothesis that distance from the ligation significantly modifies the remodeling response, we re-analyzed the data from our previously published study,¹⁵ using the alternate method of analysis described below. Interpretation of the data were initiated with scatter plots of the different vessel areas *versus* distance for each mouse, and mean values for each distance were calculated. Consistent with the description of Kumar and Lindner,⁷ while sham-manipulated vessels showed no changes in vessel areas over the length of the arterial segment, quantitative analysis of ligated arteries demonstrated changes in total, medial, intimal, and lumen area depending on the region (Figure 1C). For example, intimal area was greatest in the 1 mm closest to the ligation, and gradually decreased in the direction of the aortic arch. Conversely, lumen area was small in the 1 mm closest to the ligation, and increased with distance from the ligation. As the scatter plots for both the individual mice and groups appeared well behaved (continuous and differentiable), we considered the data suitable for regression analysis. The data, including distance from the site of ligation for each analyzed section, were coded into SAS Version 8.0. The contribution of variability with distance to the total variability was determined using Proc GLM. As changes in medial area, intimal area, lumen area, and total area all contribute to the remodeling response, separate

Figure 1. The vascular remodeling response varies with distance from the ligation and the presence of osteopontin. Medial, intimal, lumen, and total areas are shown as a function of distance from a relative start site for wild-type and OPN-null following sham manipulation (**A, B**). Medial, intimal, lumen, and total areas are shown as a function of distance from the site of ligation for wild-type and OPN-null mice harvested 14 days following ligation of the carotid artery (**C, D**). The intimal area is notably larger in wild-type mice compared to OPN-null mice. Intimal area decreases with increasing distance from the site of ligation in both wild-type and OPN-null mice. However, while a marked increase in lumen area occurs with increasing distance from the site of ligation in wild-type mice, this does not occur on the OPN-null background.

models were calculated for each using the following command: Proc GLM; Model Total, Media, Intima, Lumen = ligation distance ligation*distance/solution;

The output listed both type I and type III sum of squares which showed distance from the ligation to be a significant predictor of lumen, intima, and medial areas (Table 1). The output also provided parameter estimates for the linear regression model. With the exception of total area, these data indicated distance or the interaction of ligation with distance to significantly contribute to total variance.

Response to Carotid Artery Ligation Can Be Modeled Using Regression Analysis

We then sought to improve the models through fitting a curve to the mean response. Various functions were tested against the data for best fit. The scatter plots suggested $y = \arctan(x)$, $y = e^{-x}$, and $y = e^{-(x^2)}$ as possibilities for lumen and intimal areas. Models were determined for total, medial, intimal, and lumen area using Proc Reg (Table 2) with the following command: Proc Reg; Model total media intima lumen = ligation distance ligation*distance;

With the exception of medial area, curvilinear regression models provided substantial improvement over the linear models. For example, the curvilinear model of intimal area had an r-square of 0.5548 using a $x^{(1/7)}$ curve as compared to 0.31244 for the linear model. The linear model proved best for medial area, which was not unex-

pected, given the fact that the medial area changes very little according to distance in this model.

Regression Analysis Provides a Better Description of the Role of Specific Genes in the Response to Carotid Artery Ligation

An advantage of regression analysis is that multiple factors can be considered simultaneously. We suspected this property would be especially useful in the comparison of the response to ligation of genetic mutant to wild-type mice. To validate the utility of the method, the regression model was easily expanded with the addition of variables to consider the effect of a null mutation of the *spp1* gene (encodes OPN) on the vascular response to ligation. We applied regression analysis to a data set in which we have previously used traditional methods to show OPN affects both neointima formation and constrictive remodeling.¹⁵

As was detected previously using Student's *t*-test, the regression method identified OPN as significantly affecting the lumen area and total area before ligation (the variable OPN is a significant predictor of lumen and total area), and intimal area and total area following ligation (the variable OPN*Ligation is a significant predictor of lumen and total area). However, due to the ability of regression to assign portions of total variance to each variable, regression analysis was more sensitive than Student's *t*-test as evidenced by increased statistical sig-

Table 1. Distance Is a Significant Contributor to Vascular Injury Response

Total area					
Source	Degrees of freedom	Type I sum of squares	Pr>F	Type III sum of squares	Pr>F
Ligation	1	0.09604	<0.0001	0.00585	0.2348
Distance	1	0.00009	0.8769	0.00376	0.3398
Ligation*Distance	1	0.00869	0.1486	0.00869	0.1486
error	85	0.34774			
r-square		0.23164			
Intimal area					
Source	Degrees of freedom	Type I sum of squares	Pr>F	Type III sum of squares	Pr>F
Ligation	1	0.22187	0.0001	0.02780	<0.0001
Distance	1	0.02303	<0.0001	0.00621	0.0339
Ligation*Distance	1	0.00642	0.0311	0.00642	0.0311
error	85	0.11364			
r-square		0.31244			
Medial area					
Source	Degrees of freedom	Type I sum of squares	Pr>F	Type III sum of squares	Pr>F
Ligation	1	0.01994	<0.0001	0.00198	0.0026
Distance	1	0.00009	0.5176	0.00067	0.0743
Ligation*Distance	1	0.00103	0.0283	0.00103	0.0283
error	85	0.01755			
r-square		0.54534			
Lumen area					
Source	Degrees of freedom	Type I sum of squares	Pr>F	Type III sum of squares	Pr>F
Ligation	1	0.00039	0.6581	0.01815	0.0032
Distance	1	0.02285	0.0010	0.00189	0.3314
Ligation*Distance	1	0.01997	0.0021	0.01997	0.0021
error	85	0.16799			
r-square		0.20461			

Measurements of vascular areas were coded into SAS Version 8.0 and sum of squares calculated using Proc GLM. Distance or Ligation*Distance is a significant contributor to the total variance for lumen area, medial area, and intimal area.

nificance. Our previous results using Student's *t*-test to compare OPN-null to wild-type at 14 days following ligation were: total area, $P = 0.024$; intimal area, $P = 0.003$; lumen area, $P = 0.420$; medial area, $P = 0.040$ (compare to Table 3). More importantly, the regression method also identified an interaction between OPN, ligation, and distance in lumen area that was not previously detected (the variables Ligation*Distance and OPN*Ligation*Distance are significant predictors of lumen area). This difference is due to the fact that analysis by Student's *t*-test simply

cannot consider multiple variables (compare Figure 1, C and D, Table 3). These interactions mathematically describe the dramatic change in lumen area with increasing distance from the ligature evident in wild-type mice in Figure 1C. This change in area with increasing distance from the ligature does not occur in the lumen area of OPN-null mice following ligation. In essence, the regression analysis identified, quantified, and determined the statistical significance of a marked difference in the response to carotid artery ligation that was not detected by traditional analysis.

Table 2. Regression Model of Vascular Injury Response

Model: Area = $B_1 + B_2$ (Ligation)+ B_3 (Distance)+ B_4 (Ligated*F(Distance))				
	Intima	Total	Media	Lumen
B_1	-0.0084	0.1236	0.0365	0.0889
B_2	0.2457	0.02074	0.01915	-0.08118
B_3	<0.0001	-0.00013	-0.00005	0.00009
B_4	-0.1074	<0.0001	0.00005	0.0012
F(Distance)	Distance ^{1/7}	Distance ³	Distance	Distance ^{3/4}
r-square	0.5548	0.4027	0.5453	0.4723

Measurements of vascular areas were coded into SAS v8.0 and regression models calculated using Proc Reg. Curves were fit to each area versus distance plot using the F(Distance) indicated. All parameters demonstrated statistical significance. Ligation is a boolean variable indicating whether the mouse was ligated (1) or a procedural control (0). Distance is a continuous variable indicating distance (microns) from the site of ligation or the relative start site in procedural control animals. Distance*Ligation is a continuous variable indicating the interaction of the presence of ligation with distance from that ligation.

Table 3. Regression Model of the Vascular Remodeling Response of Wild-Type and OPN-Null Mice

A.	Model: Area = $B_1 + B_2$ (Ligation)+ B_3 (OPN)+ B_4 (Ligated*F(Distance))+ B_5 (OPN*ligation*F(Distance))			
	Intimal area	Total area	Medial area	Lumen area
B_1	0.0005	0.0762	NA	0.0576
B_2	0.0037	-0.0269	NA	-0.0342
B_3		0.0123	NA	0.0093
B_4	0.0126		NA	-0.0112
B_5	0.0193		NA	-0.0157
F	$E^{-(distance^2)}$	Distance	NA	$E^{-(distance^2)}$
r-square	0.2456	0.3091	0.0824	0.7685

B.	Intimal area	Total area	Medial area	Lumen area
B_1	0.7421	<0.0001	NA	<0.0001
B_2	0.0797	0.0001	NA	<0.0001
B_3		<0.0001	NA	<0.0001
B_4	0.0010		NA	0.0006
B_5	0.0004		NA	0.0031

The model listed above for vascular areas provides parameters for: the effect of ligation (B_2), the effect of OPN in the absence of ligation (B_3), how the presence of a ligation effects the change in vessel area with distance from the ligature (B_4), and how OPN changes the effect of ligation on the change in vessel area with distance from the ligature (B_5). As distance from a relative start site had no effect in unligated mice, the parameter for this variable is not shown. Given this model, vessel areas at any point in the arterial segment can be calculated simply by inserting data for each variable into the model. Additionally, the model equation summarizes the effects of different variables. For example, in this case the presence of OPN in unligated mice results in a larger lumen area (B_3 for lumen area is a positive number that is highly significant), and greater neointima formation in ligated mice (B_5 for intimal area is a positive number that is highly significant). A) Measurements of vascular areas were coded into SAS v8.0 and regression models calculated using Proc Reg. Area versus distance curves were fit to each area using the F(Distance) indicated. As the r-square for medial area is quite low, we do not consider the model to offer improvement over comparison of means using Student's t-test. B) Statistical significance of each parameter listed in panel A. NA, not applicable.

To clarify the need to consider distance from the ligature, we created regression models of lumen area and intimal area that did not consider distance in the comparison models. In the case of intimal area, the r-square associated with the model without distance dropped to 0.0846. Thus, consideration of distance provided a 2.9-fold improvement in the intimal model. In the case of lumen area, the r-square associated with the model dropped to only 0.7171, implying a consideration provided only a 1.07-fold improvement.

Discussion

Using a multivariate approach, we demonstrate improved analysis of the vascular remodeling response to carotid artery ligation. We show regression analysis provides increased sensitivity and description as compared to methods typically used in the analysis of carotid artery ligation data. Within the vascular area models, the presence of ligation was described with a boolean variable (variable:ligation), and the distance from the ligation was described with a numeric variable (variable:distance). The models were easily extended to compare wild-type to mice lacking a particular gene using an additional boolean variable (variable:OPN). Similarly the comparison of wild-type to mice that overexpress a particular gene product could have been done with the inclusion of a numeric variable (describes the amount of gene product present). Besides the increased sensitivity that results from the portioning of the total variance, the method allows consideration of the interactions of test variables (variables: ligation*distance, ligation*distance*OPN, etc). As the vascular response is known to involve many factors, this flexibility makes the method especially suitable. In this study interaction is best demonstrated between the

ligation procedure, the presence of OPN, and distance in the model of lumen area. The change in lumen area with increasing distance from the site of ligation is significantly greater in mice that can make OPN. Phrased in terms of the lumen model, the dependence of a distance effect on ligation in mice that can make OPN is expressed as the interaction of ligation, distance and OPN being a significant predictor of lumen area. This important aspect of the remodeling response cannot be detected with conventional analysis. The quantification of the relationships of multiple variables provided by this analysis should improve our understanding of vascular pathologies, since this method is applicable to other models as well.

Distance from the ligature, a significant predictor of the response to carotid artery ligation, is a variable common to all carotid artery ligation experiments. Inclusion of distance as a variable led to a 1.07- to 2.9-fold model improvement. Furthermore, the addition of a distance variable reveals aspects of the response that univariate methods simply cannot consider. Such information improves the description of the response as compared to previously used methods. Interestingly, it was the relatively small 1.07-fold improvement with the inclusion of distance in the model of lumen area that revealed the regional aspect of the vascular response of wild-type mice not present in OPN-null mice. Thus, even small model improvement can provide important insights into the remodeling response.

Further analysis of the data and regression models reveals that one mouse had a response to ligation that was much greater than that of the other ligated mice. Examination of the injury response in this mouse showed a large clot in the lumen in the region less than 1 mm from the ligature. This clot is probably the result of altered coagulation or a procedural problem and demonstrates

the many influences involved in the vascular response. Interestingly, the outlying data came not just from sections containing the clot, but also from sections observed to be free of clot. We interpret this as showing that the presence of the deposit alters not just the response of sections containing the clot, but the response along the entire vessel. Thus, the routine exclusion of the data from the 1 mm arterial segment closest to the ligation removes the ability to detect an important source of response variation. To determine the effect of these outlying data on the analysis we repeated the analysis with the outlying data included. Though slight decreases in *r*-square values occurred for the linear models of the different vessel areas, inclusion of the outlier data did not alter the predictor significance of each variable. However, our attempt to improve the model with curve fitting was not as successful. The curvilinear model offered little improvement over the linear model using the data set containing outlying data. Thus, the exclusion of data from mice that demonstrate an obviously atypical response to the ligation procedure is important to the development of good models of the vascular response.

The vascular response induced by carotid artery ligation is typically localized in the region from 3 mm to 5 mm region closest to the ligation. In this study the response was observed to involve approximately 3 mm. Several other models of obstructive vascular pathology involve a similarly localized response. These pathology models might also benefit from regression analysis as the responses are multifactorial and may vary with distance along an arterial segment.

As often occurs in the regression analysis of data from experiments, there are some theoretical problems in applying regression analysis to the data from carotid artery ligation experiments. Regression analysis requires observations to be independent. This condition is not met in these experiments as the response at one point in the artery is physically linked to the response at every other point. We addressed this problem with the selection of a distance between arterial points (280 μ m) over which previous work has shown substantial change can occur. Regression analysis also requires equal variance at each independent variable point. This condition was not met, as the standard deviation of each point measured was different. Although this problem can be addressed with the use of weighted regression, we found that this approach gave minimal improvement in the models. Thus, the method is not presented. Lastly, our models do not present a hierarchy of effects. We found the inclusion of non-significant variables to greatly complicate the models without providing any additional insight. Thus, the variables are not included. We consider these theoretical problems to be minimal as compared to those of typically used methods (appropriateness of mean to describe changing data, and inspection of data before analysis).

In summary, distance from the site of ligation is a significant predictor of the vascular remodeling response in carotid artery ligation experiments. By allowing simultaneous consideration of many factors, regression analysis provides both improved description of the response to carotid artery

ligation as well as increased comparative power through the portioning of the total variance to its different sources.

Acknowledgments

We thank Drs. Lee Lucas and Michael DeLorenzo (Maine Medical Center, Portland, Maine) for guidance in the statistical analysis.

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